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C. F. A. PANTIN, Sc.D., F.R.S.

JOHN R. BAKER, D.Sc.

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Volume 89 Part 2

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The Pituitary in Normal and Parasitized Roach (*Leuciscus rutilus* Flem.)

BY

T. KERR

(*Department of Zoology, University of Leeds*)

With one Plate

Introduction

THE occurrence of the plerocercoid stage of a tapeworm (*Ligula intestinalis*) in the body-cavity of the roach is accompanied by a marked regression of the gonads, and in view of the relationship of the gonads with the pituitary it seemed possible that this gland might also be involved. Histological changes in the pituitary have therefore been studied; in the first instance to determine whether there are any seasonal changes in the glands of normal roach, particularly in relation to the reproductive cycle, and then to ascertain whether the presence of the parasite induces further change.

The importance of the pituitary in relation to the gonads in teleosts is indicated by the stimulation of the gonads in *Pimelodus* by pituitary extracts of that species (Cardoso, 1934), and the degeneration following hypophysectomy observed by Vivien (1938, 1939) in *Gobius* and by Matthews (1939) in *Fundulus*. The problem could be advanced a further stage if some particular cell type can be associated with these gonad changes in the fish, since in higher vertebrates the acidophils of the distal (anterior) lobe appear to be especially associated with anabolism and growth and the basiphils with thyroid activity and gonad maturation.

Materials and Methods

Normal and parasitized roach were collected throughout the year from Thrybergh Reservoir, near Doncaster, where a high proportion of these fish are infected with *Ligula*, together with other normal fish from various sources in Yorkshire. The pituitary of the roach is attached to the brain by a delicate stalk and largely enclosed by bone and connective tissue, so that removal attached to the brain is impossible; some glands were dissected out separately therefore and their staining reactions compared with those of decalcified specimens still attached to the brain. The differences due to decalcification proved to be slight in this particular species, and to reduce the risk of damage the gland was left *in situ* and the lower half of the midbrain and the corresponding part of the roof of the mouth were removed together and treated as a unit. Various methods of fixation and staining were tried before the following routine was adopted: fixation in Bouin, decalcification in formol-nitric,

double embedding by Peterfi's method after trimming the tissues, cutting serial longitudinal sections at 6 μ and staining by Mallory's (1936) modification of his own stain. The measurements of fish given are taken from the tip of the snout to the end of the solid part of the tail, not including the tail fin.

Observations

The Pituitary as a whole in Normal Adult Fish

The general structure of the pituitary can be seen in a median longitudinal section (Pl. I, fig. 1). The three divisions of the glandular (ectodermal) component are named from front to rear the anterior glandular region (anterior lobe of some authors), the middle glandular region (*Übergangsteil* or transitional lobe), and the posterior glandular region (intermediate lobe), for reasons given elsewhere (Kerr, 1942). As is usual in teleosts the lack of bounding connective tissue allows the cell types of each region to intermingle along the borders but the regions in themselves are histologically quite distinct. A brief description will first be given of these regions and of the cell types in which variation is irregular, followed in more detail by one of the single cell type recognized in this gland whose variations are sufficiently well marked and consistent to be associated with definite physiological states. The nervous lobe shows no changes that need be mentioned.

The anterior glandular region contains acidophils, basiphils, and chromophobes. The first are variable in size, often columnar, and rather solid cells whose closely packed granules hold both acid fuchsin and orange G to a moderate degree and soluble blue to a slight degree and hence stain a dark purplish-brown with Mallory; they have a moderate affinity also for azan and iron haematoxylin. The infrequent chromophobes look like degranulated cells of the same series. Neither show variations throughout the year nor any differences in parasitized fish. The basiphils are small rounded cells, few in numbers compared with the acidophils, usually light in colour but occurring in a complete range to very deep blue and showing considerable differences in numbers and granulation from one fish to another. It has not been possible to correlate these differences, however, with seasonal or other changes and the cells appear to be unaffected by parasitism. After Mallory these cells are similar in colour to the basiphils of the next region but after Anderson's (1929) modification of this stain have a dull amphiphil appearance, suggesting a separate type.

The middle glandular region again contains acidophils, basiphils, and chromophobes. These acidophils, however, are very small cells, with the granular cytoplasm frequently forming only a little cone on one side of the nucleus, and they have a strong affinity for acid fuchsin, azan, and iron haematoxylin and a very slight one for blue. They appear more brightly staining, therefore, and in that resemble the distal lobe acidophils of higher vertebrates. Their variations appear to be irregular, but their small size and large numbers make them an unsuitable type for study. The basiphils will be described

later. The chromophobes are not numerous and have very scanty and almost colourless cytoplasm. As Bock (1928) and others have pointed out there is nothing 'transitional', in Stendell's (1914) sense, about this region.

The posterior glandular region consists also of cells which from their reactions must similarly be called acidophils, basiphils, and chromophobes. The predominant type is a lavender-coloured basiphil, a cell, however, which has also a considerable affinity for orange G, azan, and iron haematoxylin. Neither they nor the occasional chromophobes show much variation. Scattered amongst them are acidophils with much the same staining reactions as those of the middle region but distinguishable by their larger size; these occur singly or in groups and vary from scarce to quite numerous. The variations appear to be haphazard and may be noticeable even in fish from a single catch. Again, the cells of this region do not appear to be affected by parasitism.

The Basiphils of the Middle Glandular Region

These cells are scattered throughout the region with a general tendency to form irregular groups. They can be found in a complete series from cells with light-blue cytoplasm lacking discrete granules (about 5·5 μ in diameter) through cells of increasing bulk and with increasing numbers of rather small dense blue granules. This series is so complete that it does appear to represent a genuine sequence, in one or both directions; on the other hand, the linking of the degranulated basiphils to the smaller chromophobes (approximately 4 μ in diameter) with their almost colourless cytoplasm may with less certainty be achieved through infrequent cells with reduced light-blue cytoplasm.

In describing the seasonal changes which can be seen in these basiphils a starting-point may be taken in April and early May, when the gonad is at its maximum and the shedding of eggs and sperm is about to begin. The basiphils are now also at their maximum. The light-blue cells are rare, almost all cells are deeply granulated, and the largest are oval or rounded and up to about 13 μ in diameter (Pl. I, fig. 2). The high proportion of these very large cells is most characteristic of this time of year. The size and density of the basiphils give the impression in sections that their relative numbers, as compared with the acidophils, have greatly increased; actually this does not appear to be the case, but the small size and large numbers of the acidophils make accurate cell counts impossible. After breeding there is a regression of the basiphils and by late June or July they are at their least prominent. Then the proportion of degranulated and lightly granulated cells is much higher and the size of the largest granulated cells has fallen to approximately 8 μ in diameter. After July there is a slow increase in granulation and cell size so that although there is some variation, the pituitaries of similarly sized fish in September and October are normally quite distinguishable from those of July, particularly in the size and number of the largest cells. There is a further increase during the winter, culminating in April and May.

The Gonads of Normal Adult Fish

The corresponding changes in the gonads may be outlined briefly (see also Turner, 1919, for the perch, and Bullough, 1939, for the minnow). In the female the eggs are shed in May or June, by July the ovary is producing fresh oogonia, and many of these develop as primary oocytes during the summer. These oocytes in their primary growth phase, with characteristic basiphil cytoplasm, are common by September, and some occur in the secondary growth phase, with yolk droplets in a clearer cytoplasm. By late autumn both stages are abundant and many possess a vitelline membrane; little further change occurs during the winter and the final general maturation only becomes pronounced by spring. In the male in July the germ cells, characterized by their large lightly staining nuclei, are giving rise to numerous little groups of smaller denser spermatogonia; this continues into the autumn; in October primary spermatocytes appear and increase throughout the winter, but the final phases in the production of spermatozoa are again confined to the spring.

The Pituitary and Gonads of Parasitized Fish

These fish have been caught from 7 cm. in length up to 22 cm., but the commonest size throughout two complete years has been 10–12 cm. with few fish ever exceeding 14 cm.

The effect of this parasitism on the pituitary is shown only by the basiphils of the middle glandular region, but there it is distinct and consistent. These cells differ from those of normal fish in the smaller maximum size that they normally attain (up to about 6·5 μ in diameter), in their much lower general level of granulation, and in the high proportion of small specimens with more or less clear blue cytoplasm. These differences are, of course, most marked in April and May (Pl. I, 3) when the normal basiphils are at their maximum, but they remain clear throughout the year. There is some variation from fish to fish even in the same catch, such as the appearance of an occasional more heavily granulated cell, but it is not sufficient to overlap the normal condition. Seasonal changes also are either absent or so reduced that they are obscured by this individual variation. The impression given by complete sections under lower power is that there has been a great reduction in the proportion of basiphils, but detailed observation under high power does not support this and it seems more probable that the proportion is not significantly altered.

The gonads of parasitized fish show a very uniform level of development at all times of the year, and this is true not only of the very small gonad which is typical of these fish but also of the occasional larger examples that do occur. The ovaries contain oogonia and primary oocytes which have developed up to about the end of their primary growth phase, the features of the secondary phase (loss of basiphility in the cytoplasm and the appearance in it of vacuoles and yolk droplets, and finally the vitelline membrane) have not been seen in the ovaries cut. In the testes the vast majority of the cells are germ cells with large clear nuclei and only a few small groups of the darker spermatogonia appear amongst them. The nuclei of the germ cells are more uniformly large

n size than in unparasitized fish, possibly owing to the lack of transformation stages towards spermatogonia, and the internal connective tissue framework gives only a very indefinite indication of a lobular organization. In both sexes, therefore, the gonads are comparable to those of spent fish in which all the later maturation stages are missing.

Conditions in Immature Fish

The largest roach whose gonads are still immature in May are 7–8 cm. in length, and they show some features complementary to the foregoing. The ovaries with their oogonia and early primary oocytes resemble in sections those of parasitized or spent fish, although the gonad as a whole is very small; the testes, too, show a corresponding level of development except that the groups of spermatogonia are more numerous. The pituitary in both sexes is characterized by the small size of the basiphils of the middle region. These, with their reduced and lightly granular cytoplasm, closely recall the cells of parasitized fish; they occur in definite little clusters, foreshadowing the larger and less well-defined groupings seen in the adult.

Thyroids of Adult Fish

The thyroids of normal and parasitized fish were cut in May, July, October, and January. The diffuse nature of the gland and the variations in different vesicles, even indeed in different parts of the wall of the same vesicle, make comparisons unusually difficult. Only very marked differences would show convincingly and such were not observed, but it must be emphasized that a considerable range of activity is possible without the manifestation of really distinct histological effects.

Sticklebacks infected with Schistocephalus

The case of the stickleback (*Gasterosteus aculeatus* L.) infected by *Schistocephalus solidus* closely resembles that of the roach and *Ligula*—in the life-histories of the tapeworms concerned, in the occurrence of the plerocercoid in the body-cavity of the fish, and in the relatively enormous bulk of tapeworm to fish—and, since no information was elsewhere available, a brief examination has been made of such infected and normal fish. Bock (1928) did not find any typical middle-region basiphils in the normal pituitary at all; this may have been due to his employment of Susa since the use of this fixative, like decalcification, causes the basiphils to stain much like chromophobes. If the gland is fixed in Bouin, or better still corrosive formol, and dissected out with needles before sectioning, the basiphils can be seen as small, moderately staining, but not numerous cells, less well suited to the observation of changes than those of the roach, yet sufficiently clear to show any marked variations. No differences could be seen, however, between those of normal and parasitized fish. In the testes of infected fish cut in May there are appreciably more germ cells and spermatogonia still present than in normal fish, but there are at the same time large numbers of spermatozoa filling the lobules of the gland; full

sexual coloration also develops in parasitized fish and there seems little reason to doubt that they breed. In the ovaries the secondary growth phase of the oocyte, with yolk and vitelline membrane, certainly proceeds, but amongst the largest eggs there is a very marked degree of atresia (compare Vivien, 1939) and it is not possible to say whether eggs capable of fertilization are produced. However, it can be said that this is a case of much more balanced parasitism in which a pituitary of not noticeably altered appearance can carry the gonads towards the end of their maturation at least.

Discussion

The roach pituitary has its glandular component divided into the usual three regions found in teleosts—here called anterior, middle, and posterior glandular regions—and the division is based upon the cell types contained and not upon any precise connective tissue or other anatomical separation. Each region contains cell types which for descriptive purposes must be grouped under the three general headings of acidophils, basiphils, and chromophobes, but differences in size, shape, and staining reactions of the chromophils permit a clear differentiation. In the glands examined more or less distinct variations have been found amongst these chromophil cells from fish to fish and from season to season, but with the exception of those in one cell type they have been considered as too erratic or indefinite to sustain any conclusions.

The exceptional cell type is the basiphil of the middle glandular region (*Übergangsteil*, transitional lobe). When stained with Mallory these cells can at all times be found as a complete sequence from examples with non-granular pale-blue cytoplasm through stages with increasing amounts of small bright-blue granules up to large heavily loaded cells. Just before breeding in April and May the number of very large dense cells is at a maximum; after the breeding season the size and granulation decrease to a minimum in July with an increase in the proportion of degranulated cells; from then on there is a slow building-up until the pre-breeding condition is again attained. On this evidence an association between these basiphils and the maturation of the gonads can be suggested, and this suggestion is supported by the condition of the pituitary in fish parasitized by *Ligula*. Here the gonads are permanently reduced to about the level found in freshly spent fish—in the ovary oogonia and early primary oocytes, in the testis germ cells and some spermatogonia, with all the later maturation stages missing in each case—and the middle region basiphils in the pituitary are smaller and much less densely granular than those of normal fish at any time of year. Such variations as these cells show in parasitized fish appear to be individual and not related to seasonal changes, nor are they so extensive as to overlap those of normal fish. The remaining cell types in the gland show no changes as a result of the parasitism. The conditions found in the largest normal fish still immature in May are in good agreement; their gonads resemble those of parasitized and spent fish in the level of maturation attained, and their basiphils are of reduced size and granulation.

Comparisons with other fish cannot easily be made, since seasonal changes so far recorded for teleost pituitaries are few and difficult to relate to particular physiological states. The most complete is by Matthews (1936) for *Fundulus*; he divides the glandular component of the pituitary into two regions only, though Scruggs (1939) finds it composed of the usual three, and there is also some doubt as to which region Matthews's cell types actually belong. The cycles he finds, however, appear to be as follows, using the nomenclature for the regions adopted in the present paper: (*a*) a winter increase of middle region acidophils; these are large cells in *Fundulus* well adapted to show changes, whereas in the roach they are numerous and very small and regular changes were not detected; Matthews found middle region basiphils on the other hand rare and no changes were noted; (*b*) a more indefinite summer increase in the posterior region acidophils; these cells in the roach show considerable variations but of a kind too haphazard to be reliable; and (*c*) a summer and autumn increase in large basiphils which Matthews assigns to the posterior region but Scruggs, more plausibly, to the middle. Even so no parallel to the condition in the roach can be drawn. Comparison with Bock's (1928) results is even more difficult since in the middle region of the stickleback gland he found no typical basiphils at all and his seasonal changes are restricted to a spring increase in acidophil activity; finally, no comparison can be made with the results of Evans (1937). In the change from the yellow to the silver form of the eel, however, with the corresponding development of the gonads, Bernardi (1943) finds an increase in the middle region basiphils. In vertebrates higher than the fish there is some reason to associate the distal (anterior) lobe basiphils with the gonads. In seasonal changes (e.g. Hartmann, 1944, in the garter snake; Kayser, 1940, in the hedgehog) an increase in these cells coincides with gonad ripening; in development (e.g. Schooley and Riddle, 1938, in the pigeon) their final differentiation may await sexual maturity.

The removal of the pituitary in fish (Matthews, 1939, in *Fundulus*; Vivien, 1938, 1939, in *Gobius*) results after a time in an inability to form the later maturation stages of the reproductive cells or to maintain those already formed. Since this lack of later maturation stages characterizes the gonads in both sexes of these parasitized roach, it appears that here too the influence of the pituitary has been suppressed. Comparable results have been obtained from hypophysectomized mammals. In the male (e.g. Smith, 1930, in the rat) there is a clear similarity to the fish; in the female (e.g. Desaive, 1940, in the rabbit) stages in the ovary do not develop beyond that of the earliest liquid-producing follicles, a comparison with the fish is not here so simple, but both reproductive cells appear to be primary oocytes. The opposite experiment of removing the gonads to determine the effect upon the pituitary does not seem to have been performed on fish; in mammals, however (Ellison and Wolfe, 1934, 1935, in the rat), the result is an increase in the proportion of distal lobe basiphils, possibly followed by a characteristic degeneration. The reduced basiphils of the parasitized fish can hardly represent either of these

effects; in other words, the condition of the fish basiphils can hardly be itself a result of the regression of the gonads. In higher types also thyroid functioning appears to be largely under the same basiphil control, and the effects of thyroidectomy upon the pituitary resemble those of gonadectomy (Grobstein, 1938, in the newt; Brolin, 1946, in the rat), but it has not been possible to find definite parasitic effects on the roach thyroids.

The actual cause of the reduction of the basiphils in the roach is still undetermined, except that it is connected with the presence of the tapeworm or its waste products, but the effect is the virtual elimination of the gonad-stimulating hormone of the pituitary. From a brief investigation of the closely parallel case of parasitized sticklebacks it is clear that the influence of *Schistocephalus* on this fish is very much less severe, though there are indications in the ovaries of what may prove to be a slight effect of a similar nature. In the teleost, therefore, it is suggested that the middle region basiphils are particularly concerned in the maturation of the gonads—their influence becoming effective from the level of spermatogonia onwards in the male and of early primary oocytes in the female—and their maintenance at breeding-point, whether or not other factors determine the actual discharge of the gametes. These cells then would correspond physiologically to the basiphils of the distal lobe of higher types, a lobe to which the entire middle glandular region of the fish has a strong histological resemblance.

I am again in debt to Professor E. A. Spaul, D.Sc., for reading the manuscript and for his comments on the interpretations involved, and to Mr. G. Withem and Mr. W. H. Price of Doncaster and Mr. R. W. Ward of the Yorkshire Fishery Board for facilities and help in the collection of the fish.

Summary

1. A short description of the glandular component of the roach pituitary is given, from the point of view of the various cell types.

2. The seasonal variations are described in the basiphils of the middle glandular region (transitional lobe), the only cell type in this fish whose variations are sufficiently regular to be reliable, and a correspondence between these changes and the sex cycle is pointed out.

3. In roach parasitized by the plerocercoid of the tapeworm *Ligula* these basiphils are markedly reduced in size and granulation, whilst other cell types are not affected, and the gonads are also in a condition with all the later maturation stages missing.

4. The facts presented and a discussion of other work lead to the suggestions that it is these later stages of maturation which are under pituitary control in fish as in higher types, that the middle region basiphils are the principal cell type involved, and that these basiphils are comparable to the histologically similar basiphils of the distal lobe of later vertebrates.

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EXPLANATION OF PLATE

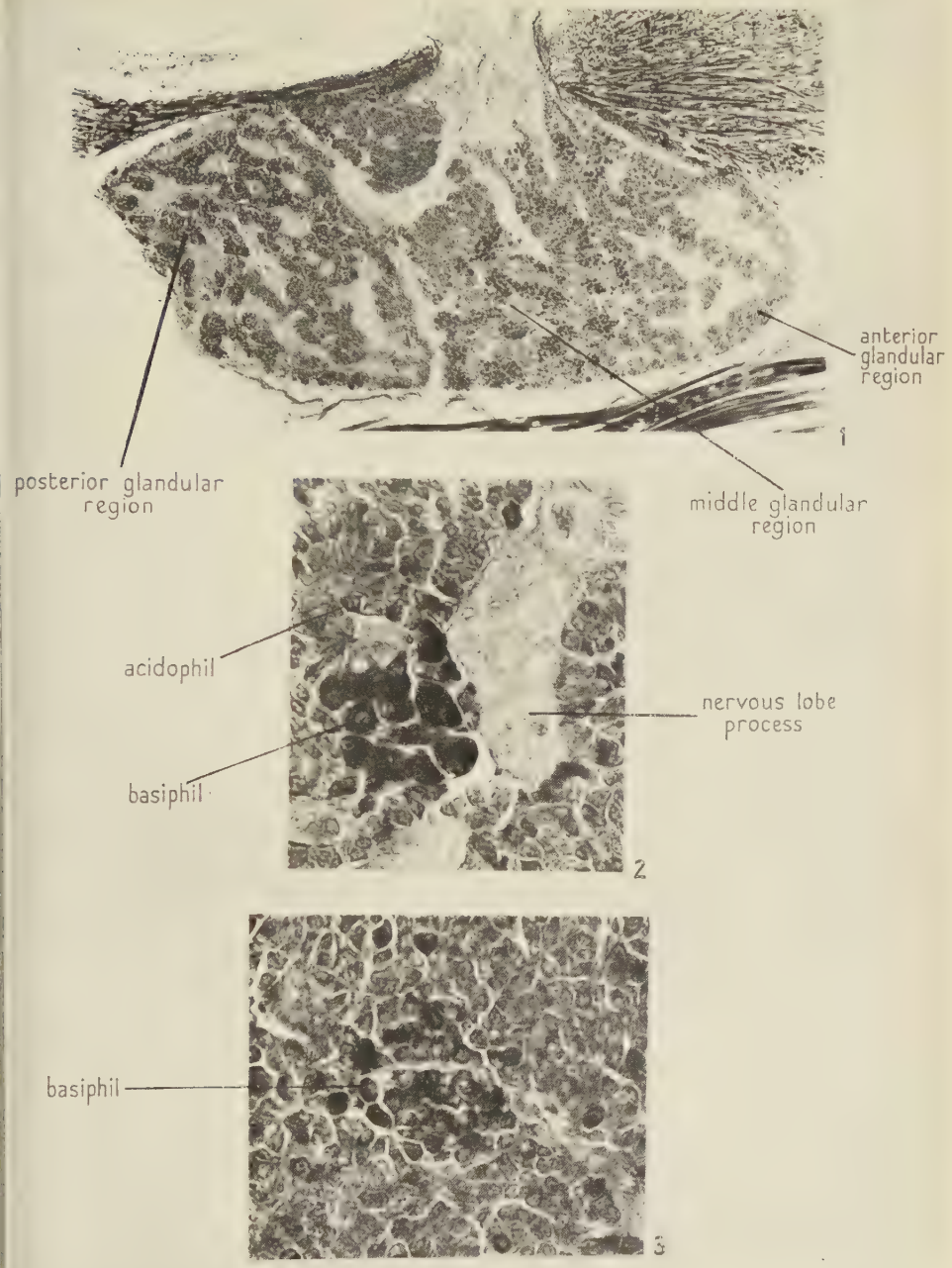
Plate I

Fig. 1. Longitudinal section of pituitary of normal roach, 14 cm. in length. May. 6 μ. Mallory. $\times 90$.

A filter has been used to accentuate the groups of bright blue basiphils in the middle glandular region. In a corresponding photograph of the gland of a parasitized fish the reduced basiphils do not show up at all.

Fig. 2. Small group of full-sized basiphils in the middle glandular region of a normal fish, 13 cm. in length. May. 6 μ. Mallory. $\times 550$.

Fig. 3. Small group of reduced basiphils in the middle glandular region of a parasitized fish, 13 cm. in length. May. 6 μ. Mallory. $\times 550$.



T. KERR.—PLATE I

An Unusual Type of Muscle-fibre

BY

JEAN HANSON

(*From the Department of Zoology, Bedford College, University of London*)

With two Text-figures

I HAVE found an unusual type of muscle-fibre in the following species of the Serpulimorpha (Annelida, Polychaeta): *Serpula vermicularis* L., *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Protula intestinum* (Lamarck), *Spirorbis militaris* (Claparède), *Sabella spallanzanii* (Viviani), *Dasychone lucullana* (Delle Chiaje), and *Potamilla* sp. The parapodia of these annelids possess bundles of bristle-like chaetae and rows of hook-like chaetae, the bristles being notopodial in the thorax and neuropodial in the abdomen. The funnel-shaped protractor muscle of the bristle bundle in both thorax and abdomen is inserted round the base of the chaetal sac and has its origin on the body-wall round the opening of the sac. The muscle consists of a large number of discrete fibres extending all the way from origin to insertion; they are not held together by any connective tissue. These fibres were dissected out from *P. intestinum* and *S. spallanzanii* and examined alive, and were studied in sections of all the species listed above.

The central part of the fibre is composed of unstriped fibrils arranged in a hollow cylinder (Text-fig. 1*a*), surrounding an axial core of cytoplasm. The cylinder is covered by a sheath of cytoplasm which communicates with the axial cytoplasm by slits in the cylinder. The sheath of cytoplasm is expanded into two or more large frill-like membranes extending along the fibre (Text-figs. 1 *b* and *c*, 2). The folding of the membranes is presumably due to the contraction of the fibril cylinder. No more than one nucleus has been found in any of these long fibres. The nucleus is situated in a thickened region of one of the membranes (Text-fig. 1 *b*, *c*, *d*). The function of the membranes may perhaps be to present a large surface-area to the coelomic fluid. The protractor muscle is poorly supplied with blood-vessels and its fibres probably rely more on the coelomic fluid than on the blood as a source of oxygen, food, &c. Fox (1938) has pointed out that the body-wall musculature of sabellids has no special blood-supply and must similarly rely on the coelomic fluid.

Not only the fibril cylinder but also the frilled membranes of the living fibres are birefringent, and when they are highly magnified in polarized light the surfaces of the membranes show a fine pattern of delicate lines orientated in all directions and crossing over each other. In sections of these fibres stained by Heidenhain's 'Azan' method a thin blue sheath envelops the fibre

and covers the surfaces of its frilled membranes (Text-fig. 1*b*). These observations suggest that the muscle-fibre is covered by a sheath of connective tissue fibres comparable to the sarcolemma of vertebrate muscles.

Apart from the frilled membranes these muscle-fibres are variants of a type of fibre commonly found in annelids (Prenant, 1929) where the nuclei are often situated on the surface of the contractile part of the fibre, and where the

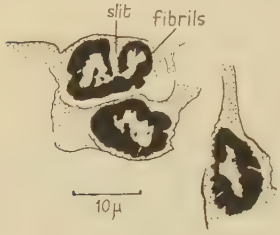


Fig. 1*a*

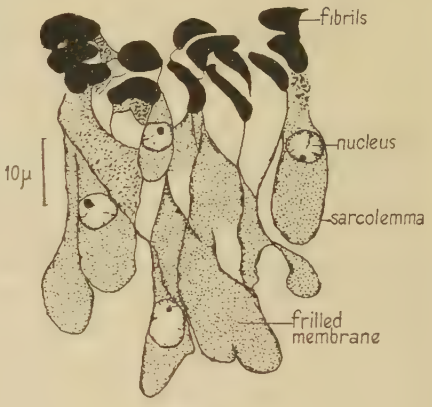


Fig. 1*b*

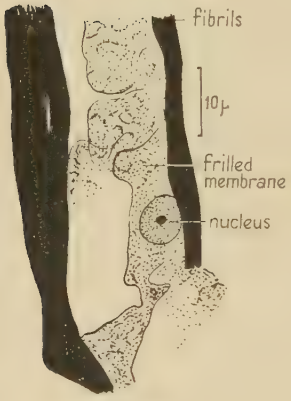


Fig. 1*c*

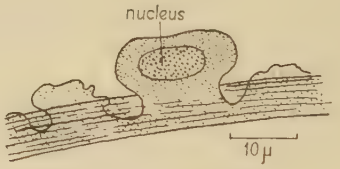
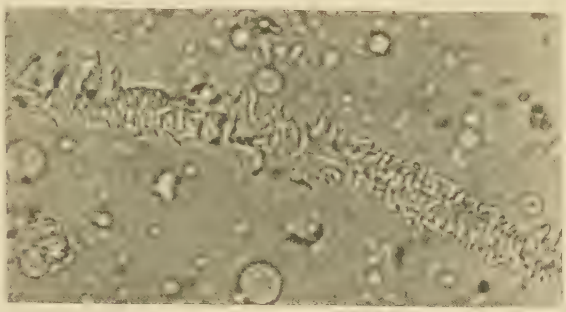


Fig. 1*d*

Text-fig. 1. Parapodial muscle-fibres. *a*. *Sabella spallanzanii*. T.S. three fibres. Iron haematoxylin. *b*. *Hydroides norvegica*. Oblique T.S. of a group of fibres. Azan. *c* and *d*. *S. spallanzanii*. *c*. L.S. part of two fibres. Iron haematoxylin. *d*. Free-hand drawing of part of fibre treated with methyl green-acetic.

fibrils are often arranged in cylinders with occasional openings putting the axial cytoplasm into communication with the peripheral cytoplasm. Goodrich (1942) isolated muscle-fibres from the 'hearts' of *Lumbricus herculeus* (= *Lumbricus terrestris* L.) and described 'processes' of the fibre which 'tend to form on either side a spreading extension with fringed edge and outstanding rounded lobes. These extensions seem to belong to a sheath of refringent material enclosing the true muscle-fibre, and it frequently shows fine closely set folds transverse to the main axis of the fibre, and giving it the deceptive appearance of a striated muscle.' I have confirmed this observation and found that the two 'extensions' are like the frilled membranes of the parapodial muscles of serpulids and sabellids.

These observations were made in the Zoological Station of Naples. I wish to thank the staff of the Station, the British Association for the Advancement of Science for the use of its Table, and the University of London for a grant towards travelling expenses.



Text-fig. 2. Photomicrograph of living parapodial muscle-fibre of *S. spallanzanii*. ×450.

Summary

The protractor muscles of the bristle-like chaetae of serpulids and sabellids consist of discrete fibres. Each fibre consists of a hollow cylinder of unstriped fibrils surrounded by a sheath of cytoplasm which is expanded into two or more frilled membranes extending along the fibre. The single nucleus lies in one of these membranes. The fibre is apparently ensheathed by a sarcolemma of fine connective tissue-fibres. Similar frilled membranes are present on the muscle-fibres in the walls of the 'hearts' of *Lumbricus terrestris*.

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The Innervation of the Muscle-spindle

BY

D. BARKER

(*Leverhulme Research Scholar, Royal College of Surgeons of England; Senior Demy of Magdalen College, Oxford*)

With three Plates and thirteen Text-figures

(*From the Department of Zoology and Comparative Anatomy, Oxford*)

Contents

Introduction

WHEN Ruffini published his classical paper on the innervation of the muscle-spindle in 1898 it was widely maintained that the tendon-jerk was not reflex in nature but due to direct mechanical stimulation of the muscle. It has since been amply proved that the jerk is a reflex phenomenon, and it is commonly supposed (see, for example, Fulton, 1946) that the muscle-spindle is the receptor which excites the muscle contraction. According to Matthews (1933) the sensory fibres which terminate as Ruffini's 'flower-spray' endings in the spindles constitute the afferent side of the arc. However, though our knowledge of myotatic reflexes is now considerable it cannot be said that our information as to the precise form and disposition of nerve-endings in the muscle-spindle has made comparable progress. It is true that through the researches of Agduhr (1919, 1939) and Cuajunco (1932) we know that the innervation may be plurisegmental, and the development of

the innervation has been studied by Sutton (1915), Tello (1917, 1922), Cuajunco (1927, 1940), and Dickson (1940). Also the regeneration of spindle nerve-fibres has been followed by Huber (1900), Tello (1907), and Boeke (1916). But in the course of these investigations no fundamental analysis has been made of the complex innervation of the normal muscle-spindle.

The 'flower-spray' ending, to which such an important role has been assigned, has only been described in detail by Ruffini himself (1897, 1898). Though a few authorities have identified endings conforming with his description (Garven, 1925; Hinsey, 1927; Cuajunco, 1927, 1932; Hines and Tower, 1928; Denny-Brown, 1928*a*), their scrutiny has not been such as to provide us with additional information about the diameter of the fibre forming the ending or the exact relationship of the ending to the intrafusal muscle-fibres and the neighbouring 'annulo-spiral' nerve-ending. Such information is essential if any satisfactory correlation is to be made with the physiological data.

The experiments of Hinsey (1927), Boeke (1927), Hines and Tower (1928), and Cuajunco (1932) have convincingly demonstrated that the spindle receives a somatic motor innervation, but no complete analysis has been made of the number and distribution of the end-plates on the intrafusal muscle-fibres, or measurements made of the diameters of the nerve-fibres taking part in this innervation. In 1932 Tower was of the opinion that 'the rich confusion of nerve-fibres at the spindle poles' still awaited thorough analysis, and the recent electrophysiological study of Leksell (1945) on the 'gamma efferent' fibres emphasizes that this need still exists.

The present investigation of the innervation of the muscle-spindle was undertaken, firstly, as a necessary preliminary to a study of spindles reinnervated after nerve-injury and, secondly, in the hope that it might provide a solid basis for theories as to the mode of functioning of the end-organ.

Materials and Methods

The recovery of the rabbit's knee-jerk after crushing and after cutting and re-uniting the crural nerve has previously been followed (Barker and Young, 1947), and an attempt is being made to correlate the degree of recovery of the reflex with the extent of the reinnervation of the muscle-spindles. The normal innervation of the muscle-spindle has been studied chiefly in spindles from the rabbit's quadriceps so as to provide a control for the experimental investigation. Some preparations were also made of spindles from the rabbit's m. interossei, and the investigation was extended to spindles from the quadriceps of the cat.

Both silver and gold chloride techniques were used to impregnate the nerve-endings. Several silver methods were tried but that which gave the best results was de Castro's modification (1925) of one of the block impregnation methods of Cajal, the muscle being fixed in chloral hydrate, alcohol, and nitric acid. Various special modifications of the buffered silver nitrate method of Holmes (1943) were tried and proved useful for the demonstration of par-

ticular aspects of the innervation. The material was embedded in paraffin, and serial longitudinal sections cut at 20–25 μ.

Although preparations were made from most of the muscles belonging to the rabbit quadriceps, the muscle most often chosen was vastus intermedius I (Bensley, *Anatomy of the Rabbit*, 1938) because of its convenient size and shape and the fact that the direction of its fibres is such as to permit easy orientation for longitudinal sectioning.

The gold chloride technique used was that advocated by Gairns (1930) and within the limitations of the method it gave consistently good results. A few spindles from the rabbit's vastus intermedius were also teased out fresh in Ringer's fluid.

A spindle successfully impregnated by the gold chloride method gives an excellent overall picture of the innervation, and as compared with fresh specimens less shrinkage appears to have taken place than in material fixed for silver methods. However, the coloration is never uniform from one end of the spindle to the other, and it is impossible during teasing to leave the origin and insertion of the intrafusal muscle-fibres intact, or to be certain that all sources of nerve-fibres taking part in the innervation have been preserved. Moreover, for a detailed study of the relationship of the endings to the intrafusal fibres and their nuclei the method is inadequate. Preparations of spindles impregnated with silver do not suffer from these disadvantages. Whilst splitting up the end-organ into half a dozen or more 25 μ longitudinal sections makes it impossible to obtain a complete picture other than by reconstruction, it is only in this way that a thorough analysis of the innervation can be made. The majority of the spindles studied were therefore reconstructed from serial sections impregnated with silver, with gold chloride preparations providing a useful supplement for observing more general features.

Many of the ideas about the innervation were obtained from a single elaborate reconstruction of a spindle from m. vastus intermedius of the rabbit impregnated by de Castro's silver method. The spindle possessed two 'flower-spray' endings which lay one on each side of a central 'annulo-spiral' termination. It occupied nine 25 μ longitudinal sections, with the equatorial region occurring in five of these and portions of the small nerve-trunk innervating the spindle lying in a further seven sections. These sixteen sections were drawn at a magnification of 750 with the aid of a camera lucida using a $\frac{1}{12}$-in. objective. The nine sections containing the spindle were each covered on an average by about a dozen fields of view; a field of view once drawn was fitted together with the previous one and in this way a complete drawing made of the spindle as it occurred in each section. Altogether 130 fields of view were drawn and fitted together. Each region of the spindle was then reconstructed by collecting and fitting together all the relevant portions on to one major tracing, a process which necessitated a great deal of re-examination and redrawing of certain portions of the spindle, notably the complex equatorial region. Each part of the reconstruction was re-checked when the final figure was drawn. From pole to pole the length of the spindle measured approximately

3·6 mm., so that at ×750 the complete reconstruction was over 8 ft. long. This has been reproduced in Plate I, reduced to a ninth of its original size.

This exhaustive study yielded much valuable information, but it was not considered necessary to make such elaborate reconstructions of other spindles. A considerable number of other spindles was examined and six spindles from the rabbit's quadriceps were reconstructed by the more rapid technique of drawing each section by eye and progressively building up a picture as the series was examined. As compared with the previous study these reconstructions were more in the nature of freehand sketches, but nevertheless they provided considerable additional information.

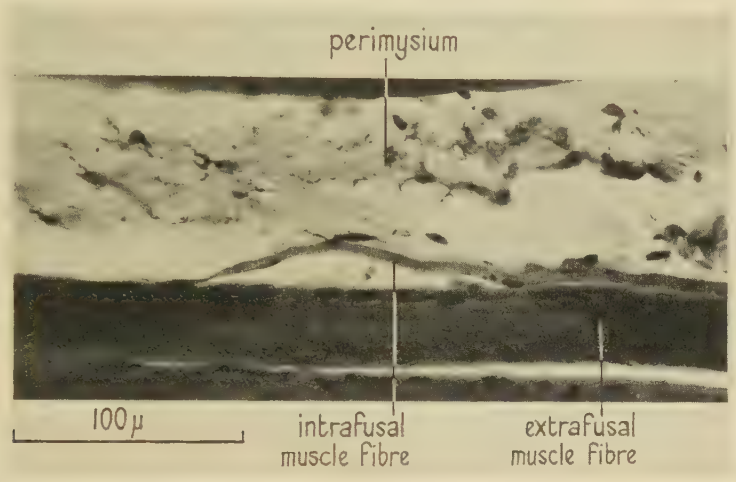
The Intrafusal Muscle-fibre

It is necessary to consider the morphology of the muscle-fibres of the spindle before describing their innervation. In the classical descriptions (e.g. Sherrington, 1894; Ruffini, 1898) a distinction is made between the two poles of the spindle; there is said to be a proximal 'muscular' pole where the intrafusal muscle-bundle begins, and a distal 'tendinous' pole where the fibres taper off in long tendinous filaments. In between the two polar regions lies the equatorial region where the muscle-bundle courses through a fusiform space bounded by a thick lamellated capsule. According to Sherrington (1894) this space is lymphatic, for he succeeded in injecting it from the lymphatics of the leg. At the extreme proximal end of the spindle two or three muscle-fibres become invested by a common sheath of connective tissue. These are 'parent' intrafusal fibres, and as they approach the equatorial region each is said to split longitudinally into two or three 'daughter' fibres. Within the equatorial region the fibres become completely filled with centrally placed nuclei and form in most cases a distinct fusiform swelling. Whilst most of these features have been repeatedly described in the literature, it has never been convincingly demonstrated that the intrafusal fibres do undergo subdivision, and there is conflict of opinion as to whether or not the cross-striations are interrupted in that region of the fibre possessing the nuclear aggregation. A resolution of both these issues is essential to an understanding of the mechanism of the contraction of the intrafusal fibres and the effect this may have upon the sensory terminations.

The divided condition of the intrafusal muscle-fibres has been reported by the majority of observers from Kühne (1863) to Denny-Brown (1928*a*). Forster (1894) described how in the spindles of man the intrafusal fibres not only divided but became reunited again in such a way that their number in the equatorial region was from twice to four times that at the poles. Such division and re-fusion was also held to occur by Batten (1897). However, Kerschner long ago (1888) doubted whether subdivision of the intrafusal fibres occurred, and Baum (1900) failed to find any sign of it in a study of serial transverse sections of hedgehog spindles. Baum showed that the number of intrafusal fibres varied as a series was followed through from pole to pole, but found this to be due to the fibres being of different lengths, some

tapering off earlier, others later, in the polar regions. Cuajunco (1927), in his study of the development of the muscle-spindle of the pig, states that in all his preparations both of embryonic and adult material the intrafusal fibres preserved their individuality, the same number entering, passing through, and emerging from the capsule.

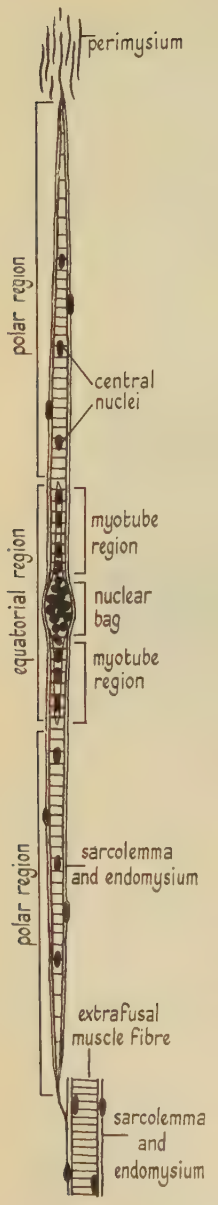
In my own preparations of rabbit muscle-spindles I have also found the intrafusal muscle-fibres undivided. The muscle-bundle is composed of 2–4 fibres and the same number can be counted throughout the greater part of the



Text-fig. 1. Extreme end of an intrafusal muscle-fibre tapering off and becoming attached to endomysium of extrafusal muscle-fibre. Rabbit, m. vastus intermedius; L.S. 25 μ, de Castro silver method.

length of the spindle. It varies only towards the ends of the polar regions where the fibres, which vary in length, arise or insert at different levels. At these points they narrow down to mere filaments with wide spacing of their cross-striations, and ultimately they taper off into thin tendinous wisps. The wisps of the shortest fibres become merged with the connective tissue sheath enclosing the small muscle-bundle, and it is the places where this occurs that often present the deceptive appearance of one muscle-fibre subdividing into two. For example, in a polar region of one spindle a short intrafusal fibre tapered off in this way 0·7 mm. from the nearest end of the equatorial region, whilst two other longer members of the muscle-bundle tapered off 0·8 mm. and 1·1 mm. farther on, becoming merged with perimysium. The ultimate ends of the longer intrafusal fibres are either attached to tendon, perimysium, or the endomysial sheaths of various extrafusal muscle-fibres in the neighbourhood (Text-fig. 1).

There appears to be no constant feature which is characteristic of one pole and not the other, such as a particular mode of attachment, a considerable difference in length, or a difference in form of the muscle-fibres. Hence it is



TEXT-FIG. 2. Diagram of a single intrafusal muscle-fibre; each polar region has been shortened to about a third of its typical length.

impossible to distinguish between the 'proximal' and 'distal' poles of a spindle by mere inspection. These terms can only be used when it is possible to orientate the spindle according to the proximal and distal ends of the muscle. This can be done in sectioned material but rarely in gold chloride preparations since the orientation is usually lost during teasing. It is convenient to retain the terms for descriptive purposes and where they are used in this paper they merely indicate the orientation of the spindle within the muscle.

Ruffini (1898) maintained that the muscle-fibres at the extreme end of the proximal pole were 'always well apart from one another, and not closely bound together as in the rest of the spindle'. This is so when the fibres are attached to the endomysium of various extrafusal muscle-fibres, for not sharing a common origin they at first necessarily course apart from one another before running together as a bundle. In many spindles the proximal pole is attached in this way with the distal pole inserting on to tendon. But I have also observed spindles with both poles attached to extrafusal endomysium, and others where the distal pole has been attached in this way whilst the proximal pole has been attached to perimysial connective tissue.

In the polar regions elongated oval central nuclei occur at widely spaced intervals within the intrafusal muscle-fibres. In the equatorial region these nuclei become numerous and lie in a rod-like core of protoplasm so as to form a continuous chain. They then give way to a great mass of smaller spherical nuclei which at one point completely fills and often distends the body of the muscle-fibre (Text-fig. 2). It is proposed to call this aggregation of spherical nuclei the 'nuclear bag' (*Bläschenspindel* of Cilimbaris, 1910) and the portions on each side of it the 'myotube regions', for it is necessary to distinguish between these parts when describing the sensory innervation. As noted by Sherrington (1894) and Cuajunco (1927), in fixed preparations the diameter of an intrafusal fibre is less in its myotube regions than at the poles. Cuajunco suggests that this may be due to pressure exerted by the lymph in the equatorial region.

Each nuclear bag contains about forty or fifty spherical nuclei and is 100–150 μ long. The bags occur in a localized region of the equatorial area, usually towards one end, and do not all lie at the same level in the muscle-bundle but in such

a way that the proximal end of one such formation may begin opposite the distal end of another belonging to a neighbouring intrafusal fibre alongside. The number of nuclear bags in a spindle is always found to correspond with the number of intrafusal fibres present and provides a useful means of checking the number as observed in the polar regions. The only exception appears to be in rare forms of compound muscle-spindles where two or three equatorial regions occur in succession at widely spaced intervals along the same muscle-bundle. The region of the bag where the nuclei attain their maximum density appears to be completely devoid of cross-striations, the contractile substance having thinned away to leave the nuclear aggregation ensheathed only by the membrane of the muscle-cell ('sarcoplasmatic membrane' of Gutmann and Young, 1944) and its sarcolemmal covering. As Cuajunco (1927) states, 'If myofibrils are present at all in this segment they must be so few in number that the alternating dark and light bands are not shown.' It seems most probable that the intrafusal fibre is a double contractile unit, its contractile polar portions being separated by the non-contractile nuclear bag. The disposition of the motor innervation supports this view (see below).

The Motor Innervation

In every spindle examined the number of motor end-plates was approximately double that of the intrafusal fibres contained in the muscle-bundle, about half the number of end-plates being situated at each polar end. Variations in this arrangement occur when one polar half of an intrafusal fibre possesses more than one end-plate. I have never observed the condition figured by Denny-Brown (in Creed *et al.*, 1932, Appendix I) where motor end-plates are located at the proximal pole only, innervated by collaterals from neighbouring extrafusal motor fibres. It seems probable that in reptiles branches of ordinary motor fibres do contribute to the motor innervation of spindles (see Bremer, 1883; Perroncito, 1901; Boeke, 1927). If such a contribution is made in the rabbit the branching must occur at a considerable distance from the spindle for the nerve-fibres taking part in the motor innervation retain their individuality far back into the nerve-trunks which carry them.

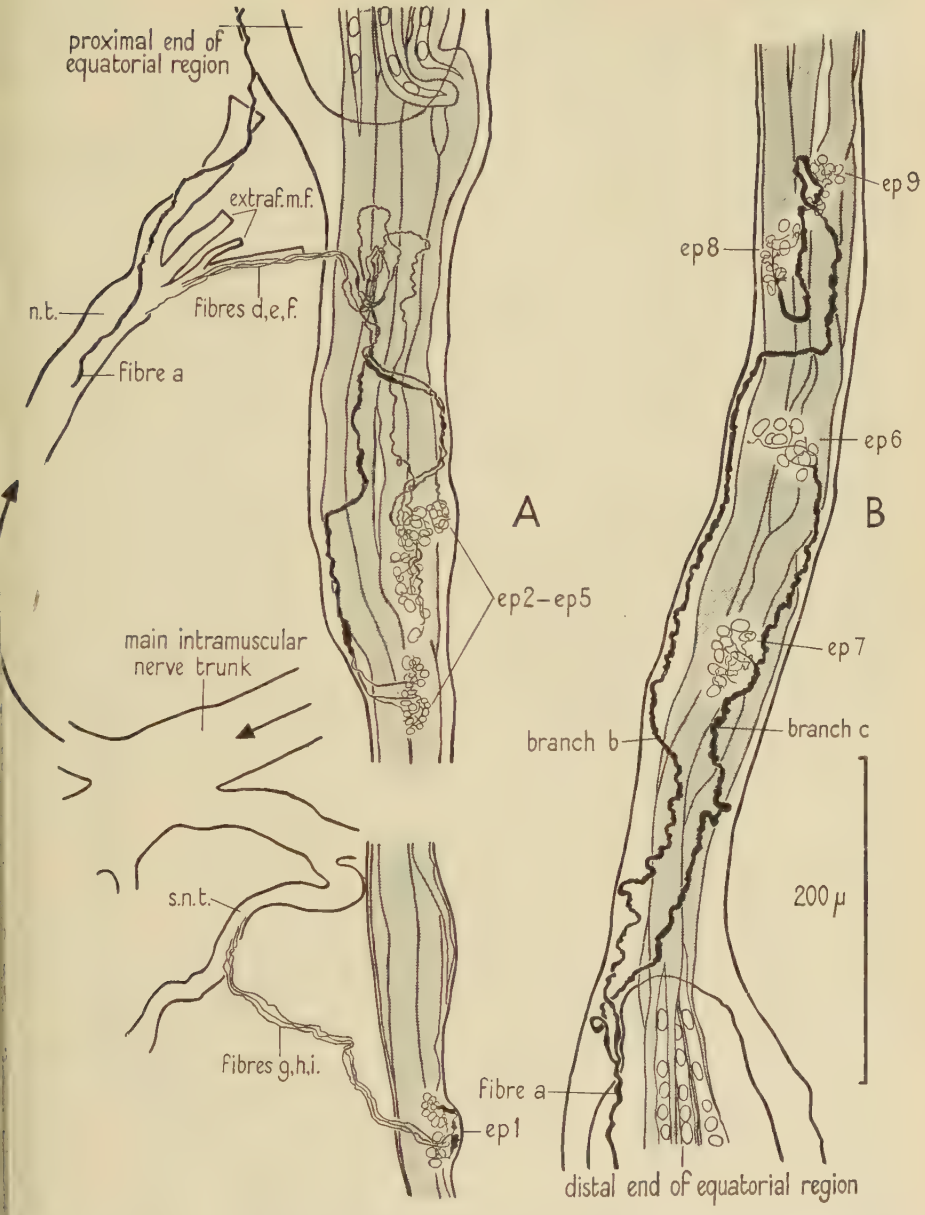
The trunk carrying the majority of the fibres innervating the spindle usually approaches the equatorial region obliquely, coursing alongside one of the polar ends, most often the proximal. The motor fibres destined for this pole leave the trunk and come into relation with the muscle-fibres. The fibres innervating the other pole usually reach the capsule with the sensory fibres and run within the capsule walls, often with a 'flower-spray' afferent, before passing on to the polar region. The spindle nerve-trunk often contains a few extrafusal motor fibres which eventually leave to form end-plates on muscle-fibres near the end-organ.

The motor innervation of the intrafusal fibres distributed in this way may be augmented by a few additional fibres which leave a small trunk of extrafusal motor fibres running near one of the poles of the spindle. In such cases

this trunk is always derived from the same mixed nerve-trunk which, lying in the neighbourhood of the spindle, supplies the rest of the spindle nerve-fibres and the surrounding extrafusal innervation.

In its general features the motor end-plate of a spindle is like an extrafusal motor end-plate (as described by Gutmann and Young, 1944), consisting of a hypolemmal termination ramifying upon a nucleated sole-plate. In silver preparations the ultimate twigs of the terminations end either as fine neurofibrillar brushes or as pointed tapers, but when treated by the gold chloride method the appearance is of blebs of various shapes for the most part connected with one another by fine filaments. A comparison between the form and disposition of the endings as seen in silver and gold chloride preparations leaves no doubt that the 'plate-endings' of Ruffini and the intrafusal motor end-plates are one and the same. The end-plates of the spindle are frequently large and often elongated so as to occupy a 50–70 μ stretch of muscle-fibre. The sole-plate may be confined to one part of the muscle-fibre or extend round the periphery to surround it almost completely. In some instances an ultraterminal twig from a large end-plate runs on for some distance to form a smaller plate on the same muscle-fibre. In such cases the endings may be regarded as two end-plates only in a limited morphological sense and are better regarded as one. On the other hand, when the smaller plate is located on another muscle-fibre it may be held to constitute a discrete termination. Nerve-fibres which enter the spindle and course without division to a single end-plate appear to be the rarest; typically the fibres divide so as to form several terminations. An end-plate frequently occurs which is innervated by branches of separate nerve-fibres or by two or three nerve-fibres which remain distinct from one another as far as they can be traced back from the spindle. It is possible that the fibres taking part in such multiple innervation are ultimately connected with a single nerve-fibre, though if this is so the branching must occur at a considerable distance from the spindle.

The motor innervation was worked out in full detail in the major reconstruction made of a spindle from m. vastus intermedius of the rabbit (see pp. 145, 146). Since examination of other spindles proved this to be a typical arrangement in most respects, it will be fully described. Text-fig. 3 shows the two poles of the spindle which possessed four muscle-fibres and altogether nine end-plates; five of these occurred on the proximal and four on the distal pole. The four intrafusal fibres could be traced through the equatorial region but in the polar regions it proved impossible to follow the course of each one with certainty. Their position in the muscle-bundle changed in such a way that although four muscle-fibres could always be detected in each section (except in the region of their origin and insertion) one was able to arrive at only an approximate idea of their complete course from one end of the spindle to the other. For this reason no strict statement can be made as to the number of end-plates possessed by any one of the muscle-fibres during its entire course, and in the figure the end-plates are not related to specific muscle-fibres. However, the

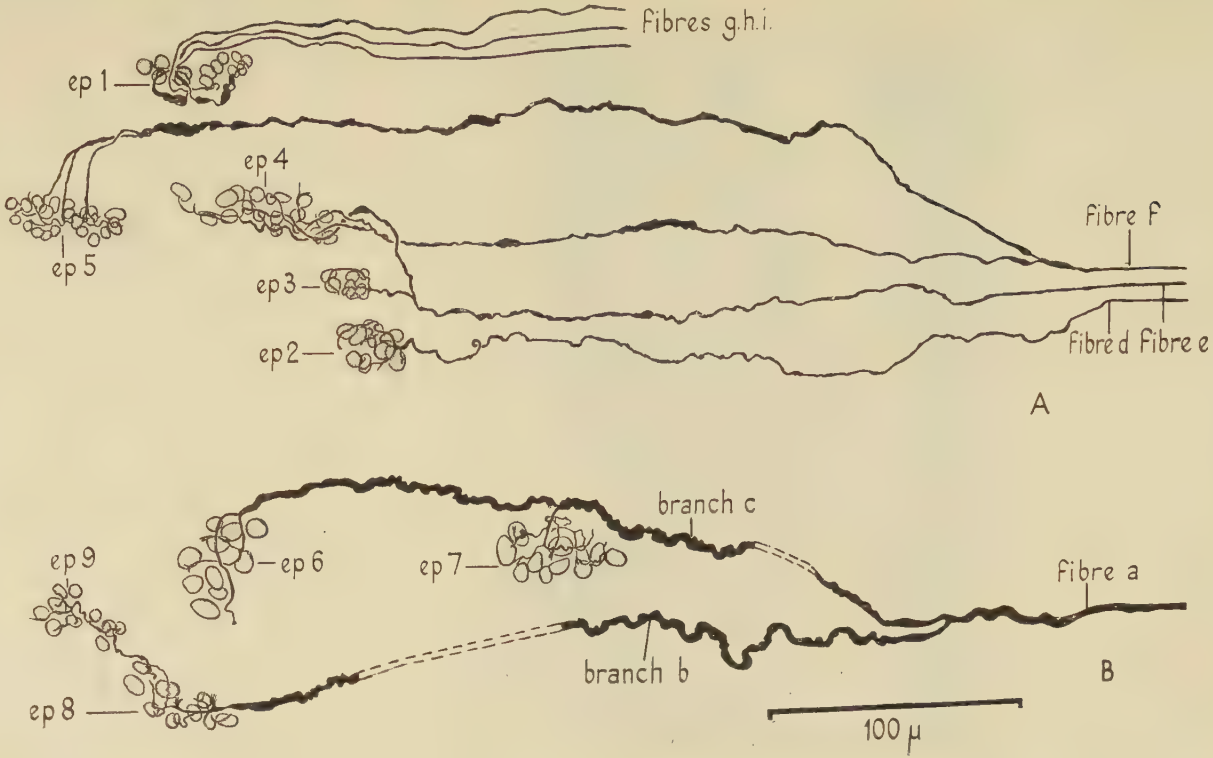


TEXT-FIG. 3. The two polar regions of the reconstructed rabbit's muscle-spindle showing the motor innervation (cf. Plate I). A, proximal pole; B, distal pole. Lettering described in text.

number and general disposition of the end-plates in this and other spindles studied strongly suggests that each polar half of an intrafusal muscle-fibre bears one, or sometimes more than one, motor end-plate, and since the two portions are separated by a non-contractile nuclear bag one would suppose that each functions as a contractile unit independent of the other.

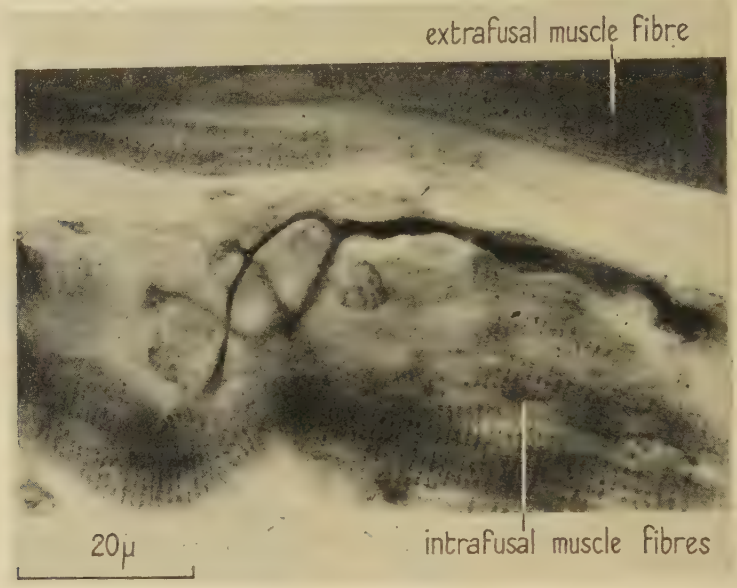
The proximal pole (Text-fig. 3A) is innervated by six small myelinated fibres; three of these (*d*, *e*, *f*) are brought in by the nerve-trunk (*n.t*) which carries seven of the ten fibres innervating the spindle besides a number of extrafusal motor fibres (*extraf.m.f.*). The other three (*g*, *h*, *i*) leave a small nerve-trunk (*s.n.t*) of otherwise extrafusal motor fibres which is ultimately connected with the same main trunk from which the other spindle nerve-fibres were derived. The total diameter of each of these small fibres in this de Castro preparation was approximately 2 μ with an axon diameter of 1 μ; measurements made of spindle nerve-fibres in fresh and gold chloride material suggest that they undergo a shrinkage of perhaps 50 per cent. in the fixed silver preparations. The three fibres *g*, *h*, *i* enter the proximal pole about half-way along its length to form a single end-plate (*ep1*) whose sole wraps round the major part of the periphery of a muscle-fibre. These three nerve-fibres retained their individuality when traced back from the end-plate for a short distance into the nerve-trunk supplying them (a total distance of approximately 0·2 mm.); they could not be followed beyond this point and it is of course possible that they represent the branches of a single fibre which divides farther away from the spindle. The three fibres *d*, *e*, *f* enter the pole near the proximal end of the equatorial region, having become segregated from the spindle nerve-trunk together with a sensory fibre. After a complex tangle over the surface of the intrafusal muscle-fibres the motor fibres course along the proximal pole to form four end-plates (*ep2–ep5*). The nature of these end-plates is shown in Text-fig. 4 where the nerve-fibres have been unravelled and the motor innervation of the two poles drawn out in schematic form. Fibre *d* (Text-fig. 4A) is seen to run without division to form a simple spherical end-plate of medium size (*ep2*). Fibre *e* gives rise to two twigs, one of which forms a small end-plate (*ep3*) close by the previous one and apparently on the same muscle-fibre, whilst the other contributes to a large elongated end-plate (*ep4*) on another muscle-fibre. Fibre *f* makes an early division into two shortly after entering the spindle; one of these branches contributes to the elongated end-plate (*ep4*) whilst the other runs farther on to form an end-plate of its own (*ep5*). All these fibres increase considerably in diameter as they approach their end-plates, or immediately prior to breaking up into the final ramifications. A fibre possessing an average axon diameter of approximately 1 μ as it approaches the spindle may thus increase up to 3·4 μ shortly before terminating. A similar increase in diameter before terminating also appears to take place in extrafusal motor fibres.

The five end-plates on this proximal pole provide a typical example of the variety that is to be found in the motor terminations of muscle-spindles. The multiple innervation of intrafusal end-plates by apparently separate



TEXT-FIG. 4. Schematic plan of the motor innervation of A, the proximal, and B, the distal pole of the reconstructed rabbit's muscle-spindle (cf. Text-fig. 3). Lettering described in text.

nerve-fibres and the occurrence of several distinct end-plates upon one polar half of a single intrafusal muscle-fibre are characteristic features, at any rate in the mammal. Cilimbaris (1910) found this to be the case in spindles from the extrinsic eye-muscles of sheep and various mammals, and both Dogiel (1902, mm. transversus and rectus abdominis of rabbit, monkey, and man) and Garven (1925, m. panniculus carnosus of hedgehog) observed several end-plates situated close together upon the same intrafusal muscle-fibre.



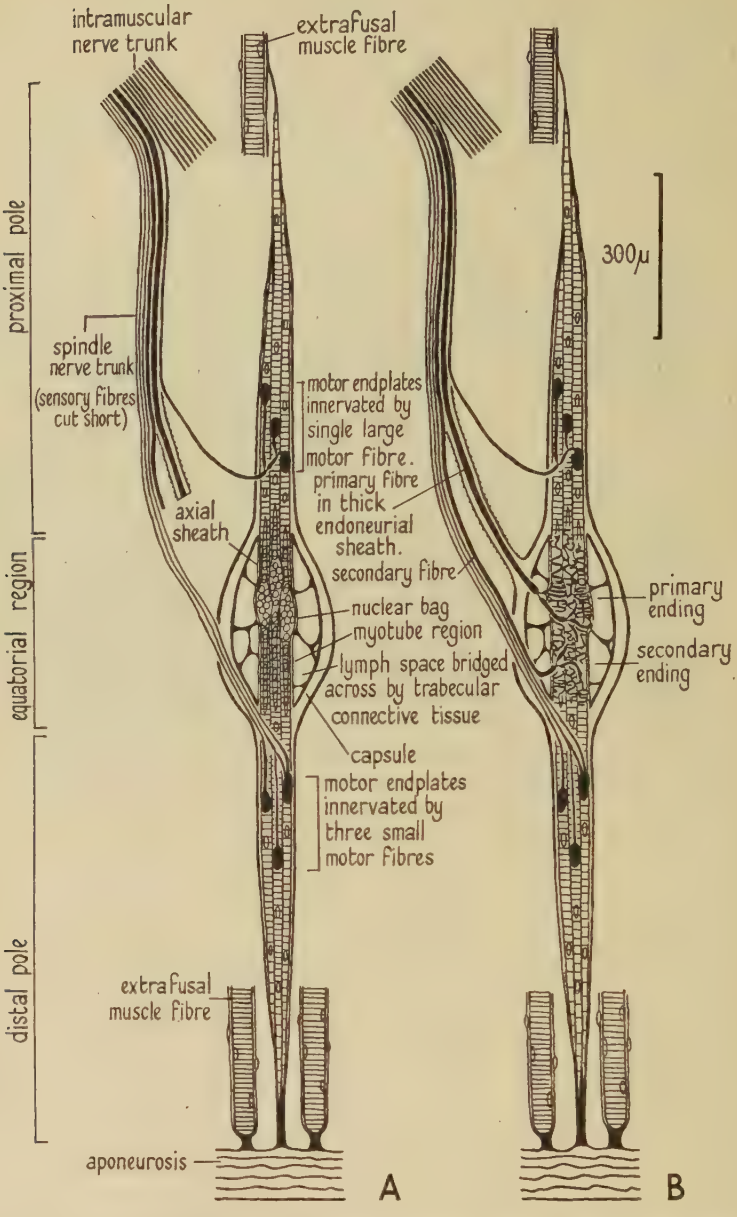
Text-fig. 5. Intrafusal motor end-plate from distal pole of reconstructed rabbit's muscle-spindle (the same end-plate as *ep6* in Text-figs. 3 and 4). L.S. 25 μ, de Castro silver method.

The distal pole of this reconstructed spindle (Text-fig. 3B) was innervated by a single nerve-fibre (*a*) which was considerably larger than any of those innervating the proximal pole. Its average total diameter was 4·8 μ with an average axon diameter of 1·8 μ. It enters the spindle together with two sensory fibres at the proximal end of the capsule. Thence it runs partly within the capsule wall, partly in the lymphatic space, finally leaving the equatorial region at its distal end branching into two as it does so. The two branches (*b*, *c*) course down the distal pole, one of them (*b*) running for the most part in the connective tissue sheath surrounding the muscle-fibres; their diameters are on an average slightly greater than that of the parent fibre. Branch *c* courses for some distance before forming the large end-plate (*ep6*) photographed in Text-fig. 5. Shortly before this it gives off a small twig which immediately forms another large end-plate (*ep7*). Branch *b* runs beyond these two terminations farther down the distal pole to loop back on itself and then run on again to form a small end-plate (*ep8*) which sends off an ultraterminal twig to

another small end-plate (*ep9*) on a neighbouring muscle-fibre. The innervation of this pole is shown schematically in Text-fig. 4B.

It often happens that one pole of a spindle is innervated by one or at the most two relatively large motor fibres rather than by a number of smaller ones. Cilimbaris (1910) observed a large nerve-fibre supplying all eight end-plates of one pole, and Garven (1925) also noted the occurrence of large motor fibres innervating the spindle. In fixed silver preparations such fibres possess a total diameter of 4–5 μ whilst when treated by the gold chloride method they appear less shrunken and measure 6–7 μ. The smaller intrafusal motor fibres are about half this size, being 2 μ or less in silver and 3–4 μ in gold chloride preparations. A motor fibre belonging to a spindle teased from fresh rabbit muscle was of these dimensions, having an average total diameter of 3·6 μ. Owing to the usual increase in diameter of the nerve-fibres before terminating, measurements made during their course over the polar regions are apt to be misleading. A representative estimate of the average total diameter can only be obtained from measurements made at various points along the fibre as it is traced back from the end-organ. It is always difficult to follow their course for any considerable distance and only in favourable instances can one make a number of measurements. In his studies of the spindles of man and the dog, Batten (1897, 1898) failed to observe end-plates, but there is little doubt that the fibres he saw running to the polar regions were motor. His measurements varied from 2 to 6 μ in the two animals (Sihler preparations; chloral hydrate, acetic acid fixation) and are thus in agreement with the above figures. Hinsey (1934), reasoning from the data of Sherrington (1894) and Eccles and Sherrington (1930), supposed that muscle-spindles were innervated by motor fibres 7 μ or less in diameter (as seen in osmic preparations). Judging from my own measurements and those of Batten this does appear to be their order of size. Moreover, the fact that the intrafusal muscle-fibres were found to be innervated in many instances by both the smaller and the larger fibres within this range is in agreement with the work of Leksell (1945) on the group of small high threshold motor fibres designated by him 'gamma efferents'. He showed that there occurred a large increase in the afferent discharge from a muscle under some initial tension when these fibres were selectively stimulated, conduction in the larger 'alpha' fibres being blocked by pressure. However, an increase in the afferent discharge could also be produced when, without a pressure block, an efferent stimulus was applied which was sub-threshold for the 'gamma' fibres and activated members of the 'alpha' group only. From other experiments he concluded that in both cases this afferent activity arose from the muscle-spindles which thus appeared to be innervated by both the small 'gamma' efferent fibres and fibres larger than these belonging to the 'alpha' group.

An idealized conception of a muscle-spindle based upon the observations that have been made on the intrafusal muscle-fibres and their motor innervation is shown diagrammatically in Text-fig. 6. The spindle possesses three muscle-fibres which course from pole to pole without division. The extreme



TEXT-FIG. 6. Diagrams of an idealized rabbit's muscle-spindle; polar regions shortened t about half their typical length. In A the motor innervation is shown, but the sensory inner vation has been omitted to demonstrate the morphology of the equatorial region. Moto end-plates represented as black disks. B shows the same spindle with the addition of sensory innervation comprising one primary and one secondary ending. Full descriptio in text.

nd of the proximal pole is attached to extrafusal endomysium, whilst at the istal pole the muscle-fibres insert on to an aponeurosis. In Text-fig. 6A the ensory innervation has been omitted in order to demonstrate the morphology f the equatorial region. A thin sheath of connective tissue ('axial sheath' of herrington, 1894) invests the bundle of muscle-fibres in this region and is nited to the inner wall of the capsule by delicate septa which bridge across he lymph space. Each muscle-fibre possesses a non-striated nuclear bag, and o either side of the bag has the appearance of a myotube. In the two polar egions the muscle-fibres each bear one end-plate so that the spindle has a otal of six end-plates with three at each pole. An exact 3 : 3 ratio is shown, for serves to emphasize the fact that in all probability the two polar halves of an ntrafusal muscle-fibre function as independent contractile units. The end-lates at the distal pole are innervated by three small motor fibres, and hose at the proximal pole are supplied by a single rather larger fibre. Spindles vith both poles innervated by large motor fibres have not been observed; in nany spindles both poles are innervated by small fibres. Leksell's experi-nents suggest that the large fibres belong to the 'alpha' group, and that the maller ones are 'gamma' efferent fibres. In the figure four motor fibres are hown innervating a spindle possessing three muscle-fibres. The number of notor fibres innervating a spindle varies considerably; it always exceeds the umber of intrafusal muscle-fibres and is often approximately double this umber. Branching of intrafusal motor nerve-fibres has only been observed fter the fibres have entered the spindle; they have always remained un-ranched in the spindle nerve-trunk when this has been traced back to its erivation from a main intramuscular nerve-trunk.

In Text-fig. 6B the same spindle is shown with the addition of an equa-orial innervation comprising two sensory terminations. The nature of the ensory innervation is described below.

The Sensory Innervation

Introduction

The sensory endings of the muscle-spindle are amongst the most compli-ated of all peripheral terminations; Sihler (1895) has described them as *ein offnungsvernichtender Anblick*. The detailed investigations of Ruffini (1893–) provide the most complete analysis that has yet been made. Huber and de Witt (1898), Dogiel (1902), Cilimbaris (1910), and Tello (1922), amongst thers, have supplied information about other mammals and vertebrates, but has yet to be correlated with Ruffini's findings. Before making such a orrelation and presenting the results of the present investigation it is neces-ary to consider Ruffini's work in some detail.

He used the gold chloride method and made the majority of his observations on uscle-spindles of the cat. He distinguished (1897, 1898) between two types of quatorial sensory termination, a primary or 'annulo-spiral' ending, and a secon-ary or 'flower-spray' ending. The primary ending is supplied by a large nerve-bre which subdivides just before penetrating the capsule. After further subdivision

the ultimate branches come into contact with the intrafusal muscle-fibres and ramify so as to encircle them with a series of rings and spirals. The endings are composed of wide flat ribbons and their turns around the muscle-fibres become more widely spaced apart towards either end of the central area of termination. At these extremities the rings and spirals give way to more irregular 'rounded clavate or leaf-like figures'. Ruffini noted that some primary endings were altogether less regular in form with only a few rings and spirals, 'the greater part showing S or C forms intercalated among forked, hooked, or comma shapes'.

The secondary ending is described as being formed by a medium-sized fibre which always enters the capsule at some distance from the entrance of the primary fibre and is usually carried in a separate nerve-trunk. Subdivision occurs only after the capsule has been penetrated, and the branches formed are never numerous. The endings always lie close to the primary ending within the equatorial region but are more dispersed. They are applied 'both around and upon' the muscle-fibres and usually consist of a great number of varicosities of various shapes joined together by fine filaments, the general arrangement often resembling a spray of flowers. Ruffini observed that some of the endings possessed 'a typical feature recalling the less common S and C claspers of primary endings'.

On the basis of the presence or absence of the secondary endings Ruffini classified the muscle-spindles of the cat into three types:

1. *Muscle-spindles with complex nerve-ending.* These possessed two secondary endings in addition to a single primary ending. The secondary endings might both be located on one side of the primary or lie on each side of it. Ruffini found spindles of this type to be the most common.
2. *Muscle-spindles with intermediate nerve-ending.* These possessed one secondary and one primary ending. The secondary ending was located on either the proximal or distal side of the primary. Ruffini found this type to be the least numerous.
3. *Muscle-spindles with simple nerve-ending.* These possessed a primary ending only, usually of the more irregular type. The muscle-fibres of these spindles were short and few in number.

A comparable study of the afferent innervation as it occurred in the rabbit has confirmed many of Ruffini's observations. Particular attention was paid to the secondary fibres and their terminations which are supposed to constitute the afferent side of the stretch reflex arc. Their distribution was found to be approximately as Ruffini maintained, but their form was not 'flower-spray' in the rabbit but 'annulo-spiral', differing only in minor details from that of the primary endings.

The Sensory Innervation of the Rabbit

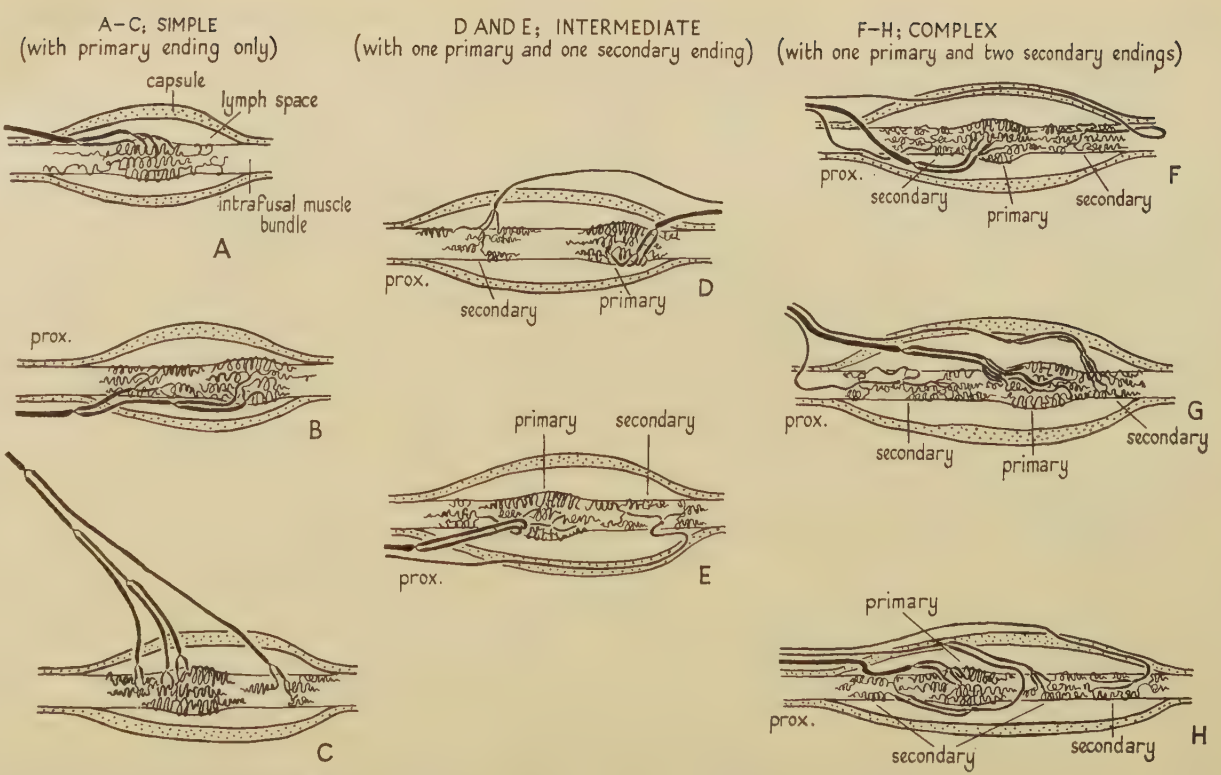
Types of Equatorial Innervation

The sensory endings are confined to the nuclear bags and myotube regions of the intrafusal muscle-fibres in the equatorial region. The primary ending is always associated with the nuclear bags, and its ramifications usually entwine only small portions of the myotube regions. In some spindles, however, particularly those in which it is the only sensory ending present, it may be

more extensive and occupy large areas of the myotube regions as well as the nuclear bags. The secondary endings are confined to the myotube regions; some of their ramifications may encroach upon the ends of the nuclear bags but never to any great extent. The two types of termination usually lie close together but never become continuous. Moreover, the fibres supplying the endings remain separate when traced back into the main nerve-trunk from which they were derived. Their course has been followed from nerve-trunk to equatorial region in a number of spindles, in one case for a distance of 3 mm., in another for 5 mm. Throughout their approach they remain as individual fibres, the primary large (8–12 μ), the secondary of medium size (6–9 μ), and there can be little doubt that this independence persists in the muscular nerve-trunks in view of Matthews's (1933) recordings from single-fibre preparations demonstrating two types of spindle afferents, and Tello's observation (1922) that the secondary fibres reach the spindle considerably later during development than the primary ones.

The disposition of the sensory endings in the spindles examined conformed to Ruffini's classification. The primary ending may be the only sensory termination present (simple type of spindle), or it may be accompanied by one secondary ending (intermediate type of spindle), or by two (complex spindle). The different types may occur within the same muscle; thus in six spindles from the vastus intermedius of a rabbit the primary ending was accompanied by one secondary ending in three spindles (intermediate type), by two secondary endings in two spindles (complex type), and in one it was the only sensory ending present (simple type). Spindles with one primary and one secondary ending occurred most frequently in the samples taken. Thus of sixteen spindles from mm. vastus intermedius and vastus lateralis of six different rabbits, ten were of this type; four others were complex with two secondary endings in addition to the primary, and the remaining two were simple with a primary ending only. A further six spindles taken from the mm. interossei of one rabbit were composed of three of the intermediate type, one of the complex, and two of the simple. In that the choice of the spindles providing these data was governed entirely by the hazards of the histological techniques employed, it can be said to be random. Providing that the impregnation was sufficiently successful to permit an analysis of the sensory innervation, the spindles were selected as serial sectioning revealed them, or as they were found by teasing.

Various types of equatorial innervation are diagrammatically shown in Text-fig. 7. Three varieties of simple spindle, where only the primary ending is present, are shown at A, B, and C; the main difference between them is the extent of the area occupied by the primary ramifications. In all types of spindle this appears to vary according to the distance between the nuclear bag region of the intrafusal muscle-bundle and the first subdivision of the primary fibre. The most usual condition is for this distance to be short, the fibre penetrating the capsule, subdividing within the lymphatic space, and almost immediately making contact with the nuclear bags. The major part of



TEXT-FIG. 7. Diagrams of the equatorial regions of eight rabbit muscle-spindles illustrating various types of sensory innervation. Intrafusal muscle-bundle shown in outline only and area of nuclear bags indicated by exaggerated swelling. Spindle C from m. interosseus, the rest from m. vastus intermedius of quadriceps. B, D–H diagrammatized from reconstructions made from silver preparations; the proximal end (*prox.*) lies to the left in each case. A and C diagrammatized from gold chloride prepara-

the ending is then situated around the bags, with a few ramifications spreading out around the myotube regions on either side. The primary ending of the simple spindle in Text-figs. 7A and 9 is of this type, and this is its typical relation to the muscle-bundle when accompanied by secondary endings. In the other types of simple spindle shown (Text-fig. 7B and C) the primary fibre undergoes its first subdivision some distance away from the nuclear bags and the area of termination is greater. The point of division occurs just before the capsule is penetrated in the spindle illustrated in B. The two branches enter the proximal end of the equatorial region; the nuclear bags, however, lie at the distal end, and between them and where the branches enter there is an extensive myotube region. One of the branches forms a group of ramifications around this region, whilst the other runs on to form another group around the nuclear bags. In the third type of simple spindle shown (7C) the primary fibre branches exceptionally early, 320 μ before penetrating the capsule. Moreover, before the capsule wall is reached one of the branches undergoes further subdivision so that altogether four separate branches enter the equatorial region. They form an extensive ending around the nuclear bag area and its myotube regions on either side. In these simple types of spindle the equatorial region is always smaller than when additional secondary endings are present, being 250–350 μ long as compared with a length of 500–600 μ in complex spindles.

Text-fig. 7D and E shows two varieties of the intermediate type of spindle where there is one secondary ending in addition to the primary; the idealized muscle-spindle shown diagrammatically in Text-fig. 6B is also of this type. The distribution of the two types of ending varies according to where the nuclear bags of the muscle-fibres are located in their course through the equatorial region. They usually occur towards one end so that the myotube regions are short on one side of them and long on the other. The secondary ending occupies the longer of the two myotube regions and may lie either proximally (as in 7D) or distally (as in 7E) to the nuclear bags and primary ending. In the spindle shown in 7F there is less difference in length between the two myotube regions for the area of nuclear bags is located more towards the middle of the equatorial region. The slightly shorter proximal myotube region receives several ramifications from the primary ending which is extensive, early subdivision of its nerve-fibre having occurred.

The secondary ending may intercalate with the primary (as in Pl. II, fig. 5) or lie well apart from it (as in Text-fig. 7D). In two instances I have observed a portion of the secondary ending extending for a short distance on to one pole, but usually the terminations are confined within the equatorial region. The primary and secondary fibres are always carried in the same nerve-trunk but course apart from each other when this breaks up at a varying distance from the equatorial region. The primary fibre usually penetrates the capsule at one end and the secondary fibre at the other or more towards the middle. The secondary fibre often courses for a considerable distance within the walls of the capsule before finally entering the lymphatic space.

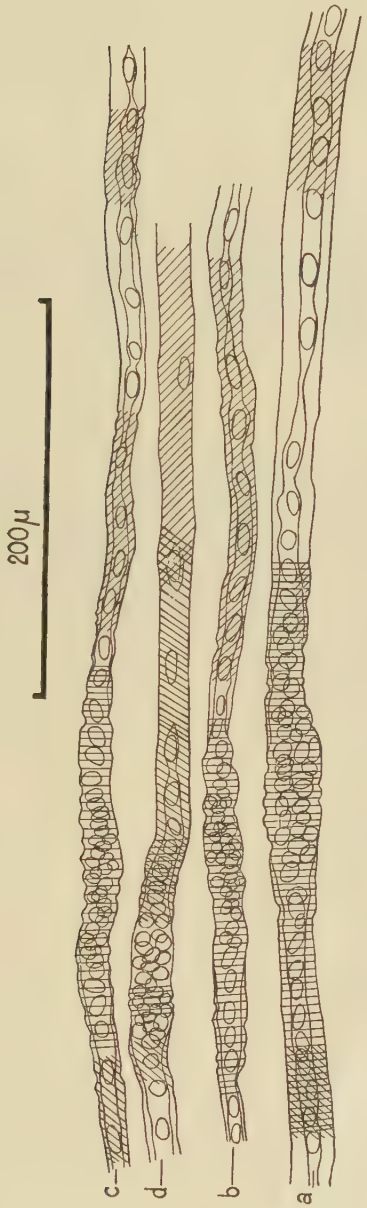
Three varieties of the complex type of spindle are shown in Text-fig. 7 F, G, and H. Here two secondary endings accompany the primary one; the most frequent arrangement is for the secondary endings to lie one on each side of the primary, which entwines the nuclear bags in the middle of the equatorial region (7F). When one myotube region is short and the other long, one of the secondary endings is accordingly less extensive than the other (7G, see also Pl. I). Alternatively, the secondary endings may lie in series with one another on the same side of the primary ending. A variation of this arrangement is shown in 7H where one of the secondary fibres divides so as to terminate not only on the distal side of the primary ending, where the other secondary ending is located, but also on the proximal side.

In the major reconstruction made of the complex spindle shown in Plate I it was possible to map out the areas covered by the three endings. Each of the four members of the intrafusal muscle-bundle could be followed from one end of the equatorial region to the other, a distance of approximately 560μ. Their equatorial portions have been accurately drawn in Text-fig. 8, where the four muscle-fibres (*a*, *b*, *c*, *d*) have, as it were, been teased apart from one another and arranged side by side. In representing the muscle-bundle in this manner the original alignment of each fibre relative to the others has been retained. The fibres vary in diameter as Cuajunco (1927) has described in the pig; the nuclear bag is greatest in the fibres of largest diameter (cf. fibres *a* and *b*). The areas occupied by the terminal ramifications of the three endings are shown by differential shading. Of the two secondary endings which lie on either side of the primary, the proximal one is the more extensive owing to the location of the nuclear bags within the distal half of the equatorial region. The primary ramifications are seen to be confined chiefly to the nuclear bags though they also extend over the distal myotube portions of the muscle fibres *a* and *b*. At four places they overlap with secondary terminations but not to any great extent. The two secondary endings for the most part occupy their respective regions on either side of the primary ending, being distributed mainly over the myotube portions but with encroachments here and there on to the end of a nuclear bag. A ramification of the distal secondary ending, however, extends over to the proximal myotube portion of one of the muscle-fibres (*d*). Here it overlaps with part of the primary ending and also with a part of the proximal secondary ending.

This map of the distribution of the three endings shows that though the areas they occupy are closely intercalated, the extent to which they actually overlap is small. The muscle-fibres are shown with marginal indentations occurring from place to place; these are constrictions caused by the nerve-endings whose form will now be described.

The primary ending

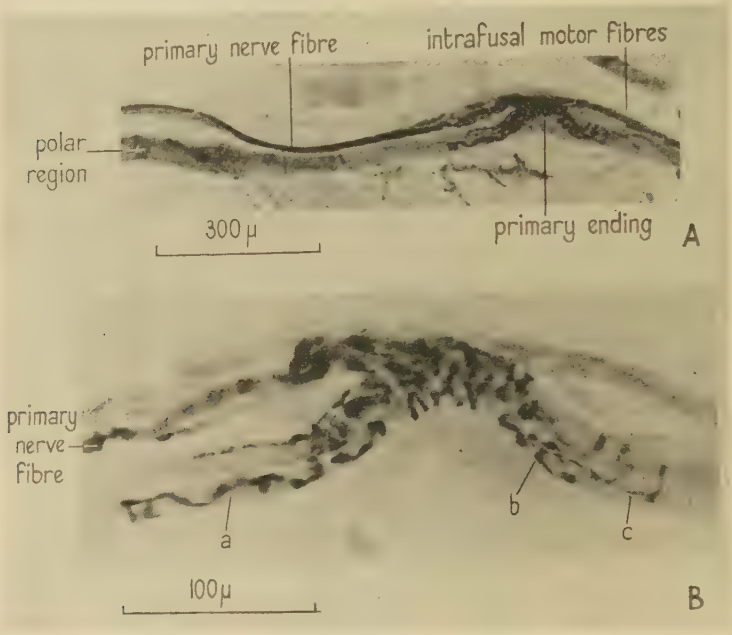
The primary ending is always derived from one nerve-fibre; I have never observed two independent fibres contributing to the termination as Ruffini (1898) found in some spindles of the cat. As noted above (p. 159), the



Text-fig. 8. The equatorial portions of the four intrafusal muscle-fibres (*a–d*) of the reconstructed rabbit's muscle-spindle. The muscle-fibres are arranged schematically side by side and the areas occupied by the primary and secondary nerve-endings are shown by differential shading. See text.

|||| primary ending, \\\\ proximal secondary ending, //// distal secondary ending.

area covered by the ending varies according to whether the first subdivision of the primary fibre occurs before or after it penetrates the capsule. When the division occurs after, the ramifications are restricted chiefly to the nuclear bags of the muscle-fibres, with only a few extensions around the myotube regions (Text-fig. 9B). This type of distribution will be considered first since it is that which most frequently occurs.



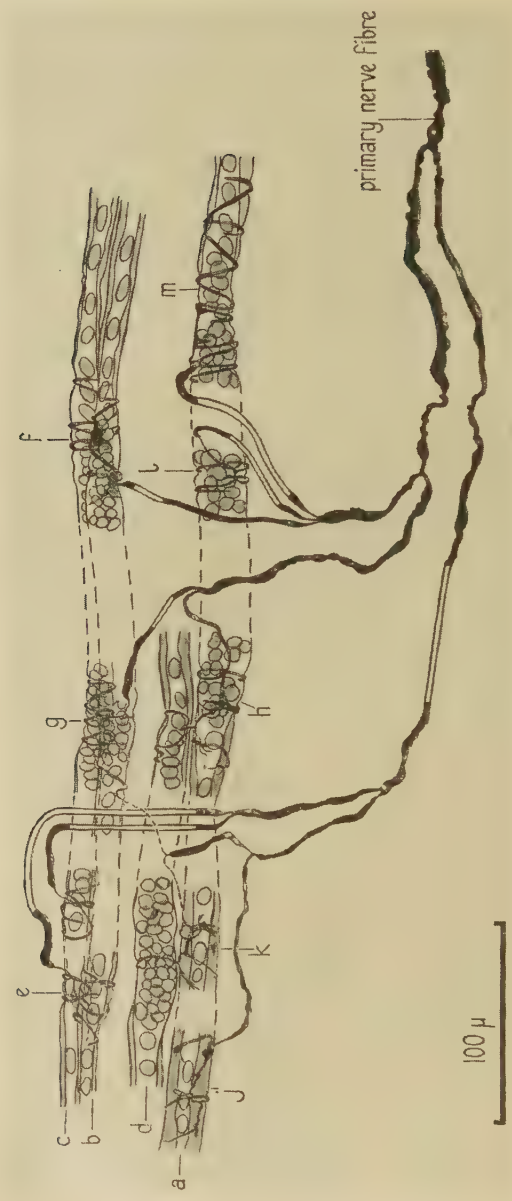
TEXT-FIG. 9A. Simple spindle with sensory innervation composed of one primary ending. B. Primary ending of same spindle. The ending is confined chiefly to the nuclear bags of the intrafusal muscle-fibres with a few ramifications (*a*, *b*, *c*) extending around the myotube regions on each side. Rabbit, m. vastus intermedius; Gairns gold chloride method.

The first division of the primary fibre is usually dichotomous, though sometimes three branches are formed. Further subdivision rapidly ensues to give rise to branches of the third and fourth order which form the terminal ramifications. These branches remain myelinated until the 'preterminal node' of Ruffini (1898) is reached and the terminations are formed. Each branch usually gives rise to one system of ramifications, though occasionally it may form two. In some instances two branches will contribute to form one system. The endings consist of a series of half-rings and spirals; complete rings are rare. They are closely set together around the middle portions of most of the nuclear bags, but to either side they are more widely spaced apart. Thus when entwining the most densely nucleated region the turns of a spiral will be vertical and tight together, but when coiling around one side of a bag and extending into the myotube region the turns are loose and run diagonally (Pl. III, fig. 10). The few terminations which extend for any distance around

the myotube regions are usually of an irregular or clasping form (Text-fig. 9B, *a*, *b*, and *c*).

A ramification system may be confined to one member of the muscle-bundle, or entwined chiefly around one but with a minor part around another, or more or less equally spread over and interlocked around two neighbouring intrafusal muscle-fibres. Each member of the muscle-bundle is entwined to a greater or lesser extent. The axoplasm wraps around the muscle-fibres in the form of a wide flat ribbon as Ruffini described. In silver preparations the ribbons have a coarse neurofibrillar appearance and the ultimate end of any termination is by means of a brush-like neurofibrillar expansion. The endings lie underneath the sarcolemmal and axial sheaths and are closely applied to the 'sarcoplasmatic membrane' of the muscle-fibres. In fixed silver preparations this relationship is particularly well shown, for where the endings curl round the muscle-fibres they constrict them so as to form marginal indentations, leaving the connective tissue running above (Pl. III, fig. 6). It seems probable that such constrictions are due to a difference in the degree of shrinkage during fixation undergone by the axoplasm of the nerve-endings and the sarcoplasm of the muscle-fibres. The muscle-fibres are not constricted in this way in fresh preparations, where in favourable instances it is possible to see spiral terminations, and in gold chloride preparations the constrictions are also absent.

In the major reconstruction made of the complex spindle (Pl. I) the primary ending was found to be composed of nine separate ramification systems. These have been schematically arranged in Text-fig. 10 so as to demonstrate the morphology of the ending to the best advantage. The figure is schematic in that the muscle-fibres and nerve-branches have been broken from place to place in order to reveal all parts of the ending. The portions thus disintegrated have been accurately drawn, and in arranging them in the form of a plan their original relationships with each other have been preserved as far as possible. The majority of the ramifications are seen to be entwined around the largest muscle-fibre present in the bundle (*a*), and this is a typical condition. The muscle-fibre *d*, on the other hand, receives only a few half-rings and the greater part of its nuclear bag is not entwined. At *e* and *f* the interlocking type of ramification system binds two members of the muscle-bundle together by means of a number of half-rings and spirals. At *g* and *h* the ramifications are distributed from a central rib somewhat as Ruffini figures (1898, fig. 5). The terminations *j* and *k* are of the irregular clasping type which is characteristic of the extremities of the ending; *j* contains a small ring lying on the surface of the muscle-fibre. At *l* three half-rings are closely set together around the middle of a nuclear bag, whilst at *m*, which lies adjacent, the terminations are at first set closely together and then form a series of loose spiral turns extending away from the nuclear bag. Spiral systems never exceed four or five turns, and when they attain this number the coils are always widely spaced and found to one side of a nuclear bag, as figured here and shown in Pl. III, fig. 10. Altogether the primary ending entwined the four muscle-



Text-fig. 10. Schematic plan of the primary ending of the reconstructed rabbit's muscle-spindle. *a–d*, intrafusal muscle-fibres; *e–m*, portions of the ending referred to in text.

fibres with a total of twenty-two half-rings, three complete rings, and twelve spiral turns. This estimation is approximate in so far as it is an arbitrary matter as to how the more irregular portions of the ending may be classified.

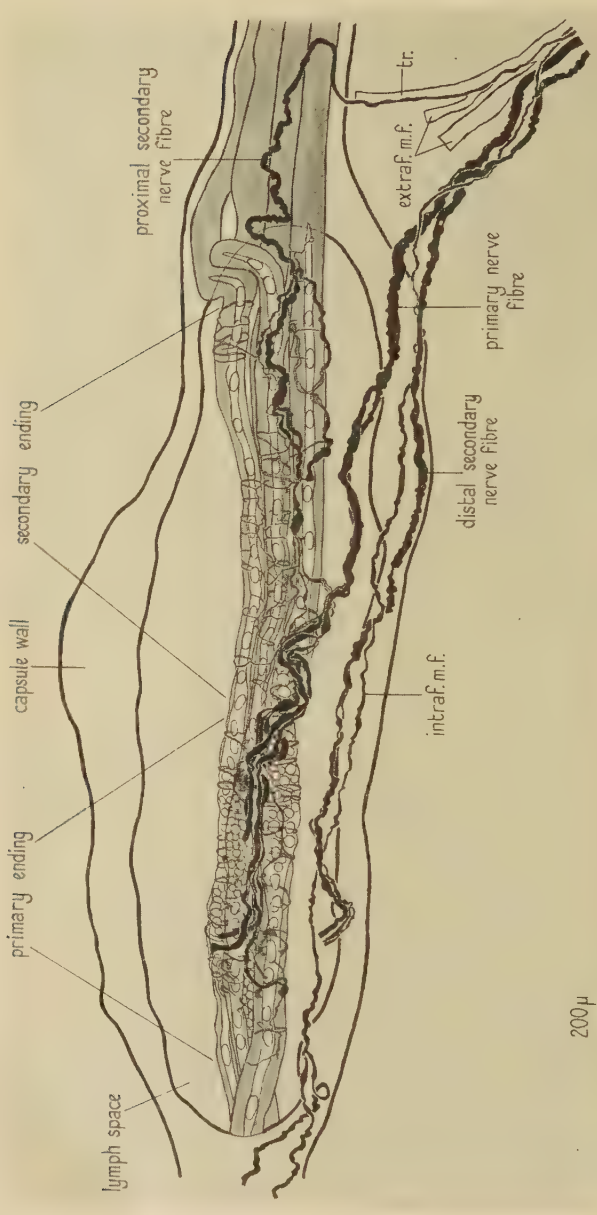
In extensive types of primary ending (as shown in Text-fig. 7B and C) formed after an early subdivision of the primary fibre, that portion of the ending which occupies the myotube regions differs little from the rest. The ramifications tend to be composed of bands thinner than those surrounding the nuclear bags, and are not usually set so closely together, but their form is the same. For the most part they consist of half-rings set at right angles to the longitudinal axes of the muscle-fibres. More irregular clasping forms usually occur as the muscle-fibres pass out of the equatorial region and the limit of the area of termination is reached. Spirals occur but not in the loosely coiling diagonal form characteristic of the nuclear bag portion of the ending.

The finding of one sensory fibre dividing so that one branch forms a primary ending in a muscle-spindle, whilst the other terminates in an adjacent tendon-organ, has been reported to occur in various mammals by Kerschner (1888), Weiss and Dutil (1896*a*, *b*), Dogiel (1906), and Hines and Tower (1928). I have frequently found that one pole of a muscle-spindle (usually the distal) lies in association with a tendon organ. In four cases of such association the sensory fibres innervating the two proprioceptors were traced back as far as possible from their terminations. They were found to remain as independent fibres, and in two cases their approach was from different directions.

The Secondary Ending

In the rabbit the form of the secondary ending differs only in minor details from that of the primary ending and the alternative term 'flower-spray' cannot be applied. So close is the resemblance that, in many cases, if the ramifications were to be judged solely by their form it would be difficult to decide whether they were derived from a secondary fibre or were part of an extensive primary ending such as is shown in Text-fig. 7B and C. The ending is, in fact, 'annulo-spiral' and consists for the most part of half-rings, rings, and spirals.

Subdivision of the secondary fibre was always found to occur after the penetration of the capsule, as Ruffini described. Some of the branches often trail for a considerable distance before terminating. The terminal ramifications, like those of the primary ending, may be confined to one muscle-fibre or spread over and interlock around two lying side by side. Each intrafusal fibre in the muscle-bundle usually bears a portion of the ending. The terminations are closely applied to the 'sarcoplasmatic membrane' and curl round the myotube regions of the muscle-fibres. In fixed preparations their coils form constrictions as in the case of the primary ending. The various systems of ramification are generally more dispersed than those of the primary sensory fibre and the flat ribbons of axoplasm are often narrower. In silver

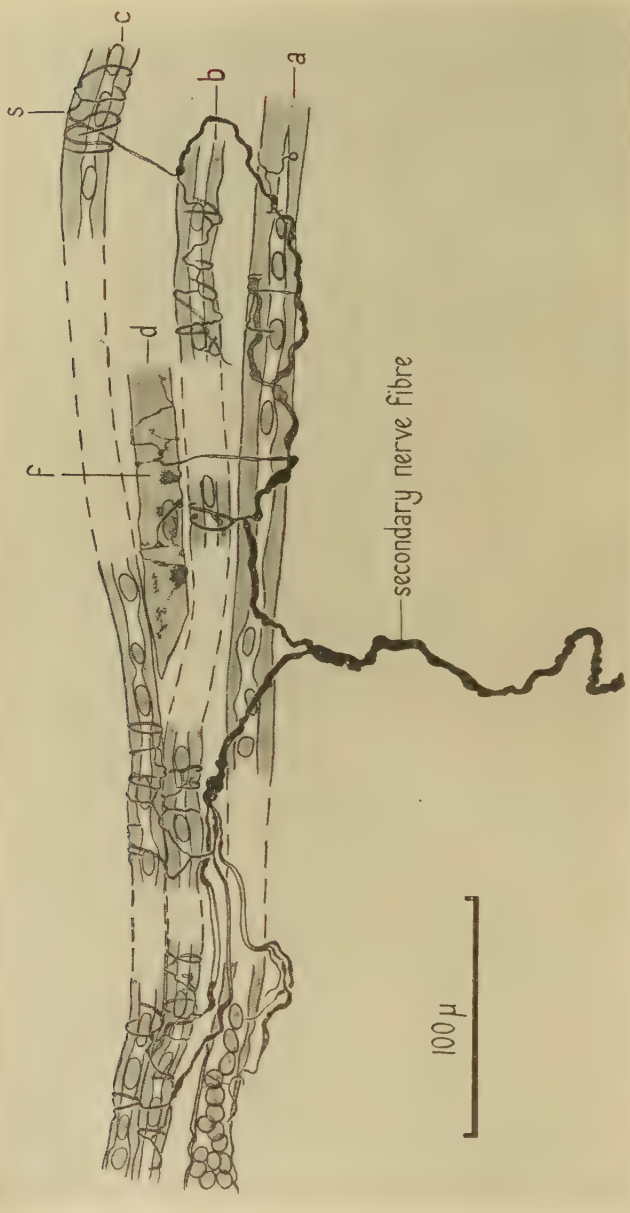


Text-fig. 11. Equatorial region of the reconstructed rabbit's muscle-spindle. The distal secondary ending and such features as the axial sheath and trabecular connective tissue in the lymph space have been omitted to avoid confusion. *intraf.m.f.*, large intrafusal motor fibre innervating distal pole of spindle (see Text-figs. 3B and 4B, fibre *a*); *extraf.m.f.*, truncules carrying extrafusal motor fibres; *tr.*, truncule carrying proximal secondary nerve-fibre and motor fibres innervating proximal pole of spindle.

preparations they appear as fine tendrils rather than wide strands of neurofibrils, and wrap around the muscle-fibres so as to form a delicate tracery rather than a set of coarse bands.

Pl. II, fig. 5, shows the primary and secondary endings of an intermediate type of spindle from the vastus intermedius of a rabbit (gold chloride preparation). The secondary ramifications are seen to entwine the muscle-fibres in much the same way as those of the neighbouring primary ending. Whilst the primary ramifications are closely heaped together and occupy approximately 160 μ of the equatorial region, the secondary ending is more scattered and covers a length of approximately 480 μ. The area covered is not always so extensive, but the terminal ramifications are invariably loosely rather than closely associated. Their 'annulo-spiral' nature is further illustrated in Pl. III, fig. 9, a photograph of the major portion of one of the secondary endings of a complex spindle impregnated with silver. The secondary terminations are seen to coil and spiral around the muscle-fibres in enlacing systems. Complete rings occur but they are uncommon; two are clearly seen in Pl. III, fig. 8, which illustrates part of a secondary ending from another complex spindle.

The similarity between the primary and secondary terminations is clearly demonstrated in Text-fig. 11, a drawing of the equatorial region of the complex spindle which was reconstructed as described on pp. 145, 146. The primary ending is shown together with the secondary ending which lay on its proximal side; the distal secondary ending has been omitted to avoid confusion. The secondary fibre enters the equatorial region and courses for some distance over the muscle-bundle before the first dichotomy occurs. Further branching eventually gives rise to a number of separate ramification systems which entwine the myotube regions of the four muscle-fibres. The ending has been schematically represented in Text-fig. 12, following the procedure that was adopted in the case of the primary ending (cf. Text-fig. 10). One of the terminal ramifications (*f*) could be said to be 'flower-spray' in form. It consists of a few fine tendrils which wrap around a muscle-fibre and at three places give rise to large leaf-like expansions of neurofibrils. The tendrils then disappear and break up into scattered clusters of granules. In some places these granules appear to be orientated with the cross-striations of the muscle-fibre, and it is difficult to decide whether they are part of the ending or a silver precipitation artifact. I have found similar ramification systems in a few other silver preparations of secondary endings. They have always occurred around a region of a muscle-fibre which, though equatorially placed, was not of the characteristic myotube form, for the central core of protoplasm was lacking and the central nuclei were few in number (as in Text-fig. 8, muscle-fibre *d*). It seems probable that such ramifications are the silver counterpart of similar irregular formations occasionally seen in gold chloride preparations (see *f.s* Pl. II, fig. 5). However, this is never the predominant mode of termination; the ramifications typically resemble the rest of those drawn in Text-fig. 12. The spiral complex (*s*) fortunately occurred intact in one



Text-fig. 12. Schematic plan of the proximal secondary ending of the reconstructed rabbit's muscle-spindle. *a–d*, intrafusal muscle-fibres; *f*, *s*, portions of ending referred to in text.

ection so that it was possible to demonstrate it photographically by optical ections (see Pl. III, fig. 7).

The Diameters of the Sensory Fibres

The primary fibre runs within a wide (15–25 μ) endoneurial sheath. It is he largest nerve-fibre innervating the spindle, with a total diameter falling vithin the range 8–12 μ (as estimated from measurements made on fresh, ilver, and gold chloride material). Batten (1897, 1898) also found the primary ibres to be of these dimensions, his measurements being 8 and 10 μ in man, nd 8 μ in the dog (Sihler preparations). The secondary fibres possess a smaller liameter which ranges between 6 and 9 μ, and they run in a narrower endo- neurial sheath. Typically the total diameter of a secondary fibre is equivalent o that of the axon diameter of the primary fibre innervating the same spindle.

The sensory fibres undergo a considerable increase in diameter as they pproach their first subdivision; for example, one primary fibre increased from 0·8 to 12·6 μ, another from 12·6 to 18 μ. As Sherrington (1894) noted, the in- rease is due to a broadening of the axon whilst the myelin sheath becomes hinner. The same holds true for the sensory fibres innervating the tendon- rgans, which otherwise are of the same dimensions as the primary fibres 8–12 μ). Ruffini (1898) states that the primary fibre is larger than that inner- ating the tendon-organ, but he does not give any measurements. Sherrington 1894) gives 7–18 μ as the diameter range of sensory fibres innervating the nuscle-spindle. However, when specifying the total diameters of two primary ibres in his description of the sensory innervation, he gives measurements f 8 μ and 12 μ (cat and monkey, gold chloride preparations). I have never bserved sensory fibres approaching 18 μ calibre innervating either muscle- pindle or tendon-organ.

The Sensory Innervation in the Cat

The primary ending in the cat, as seen in spindles from the quadriceps, as a strikingly regular appearance; the ramifications resemble a number of ightly coiled springs entwined around the nuclear bags of the muscle-fibres. Apart from their more regular disposition, however, the ramifications bear he same relationship to the muscle-fibres as in the rabbit. To either side of he central area of termination more irregular and widely spaced systems ccur as Ruffini described (1898). The ending is usually of greater size than he primary of the rabbit, since in the rabbit the number of muscle-fibres in a pindle rarely exceeds four, whilst in the cat six or more are usually to be ound. In simple spindles, as Ruffini observed, the ending is usually slightly ess regular in form. The diameter of the primary fibre, as measured in gold hloride preparations, most often lies between 8 and 12 μ as in the rabbit, hough I have found one measuring 14·8 μ.

The secondary ending, on the other hand, is more irregular than its counter- art in the rabbit. The muscle-fibres are entwined with spirals, half-rings, nd clasping systems which tend to form a background for the 'flower-spray' ype of ramification. Some of these 'sprays' spread over the surface of the

muscle-bundle and are not related to particular muscle-fibres. However, tl ending is never wholly 'flower-spray' and it is possible that Ruffini exaggerate this characteristic in his efforts to establish the existence of two types termination. The ending most often resembles Ruffini's drawing of a irregular type of primary ending (1898, fig. 3), where a number of loops an spirals are shown amongst tree-like ramifications. Tello (1922) evident found the ending to be more of this form in material from 30-day-old c embryos; his term 'claw-like ending' (*krallenförmige Endigung*) would b more expressive of the general mode of termination if a descriptive term we required. The diameter of the fibre forming the ending varies, as in th rabbit, between 6 and 9 μ (measurements made on gold chloride material).

Pl. II, fig. 1, illustrates the general appearance of the two types of endin in the cat as they occurred in a complex spindle from m. vastus medialis (go chloride preparation). In Pl. II, figs. 2, 3, the primary and secondary enc ings are shown at a greater magnification. Spindles with one prima and one secondary ending did not occur in the limited number of spindl examined, in contrast to the distribution in the rabbit's quadriceps and inte ossei where this type appears to be the most numerous. Complex spindl (with one primary and two secondary endings) were the most frequent, an this agrees with Ruffini's finding. In eight spindles from mm. vastus medial and subcrureus, five were complex and three simple.

The Use of the Terms 'Annulo-spiral' and 'Flower-spray'

When it is desired to distinguish between the two types of sensory endin present in muscle-spindles the terms 'annulo-spiral' and 'flower-spray' a commonly used in preference to Ruffini's alternatives 'primary' and 'secon dary'. However, in the rabbit the descriptive terms are not applicable, sinc both endings have an 'annulo-spiral' form. Moreover, it is evident from th literature that either termination may exist in the 'annulo-spiral' or 'flowe spray' form according to the animal in which it occurs. In the frog ther appears to be only one sensory ending present, and its association with th nuclear bags of the intrafusal muscle-fibres suggests that it corresponds to th primary ending in mammals. According to the observations of Cajal (1888) Dogiel (1890), and Huber and de Witt (1898) on frog muscle-spindles, thi ending consists of a number of varicose threads running parallel to the muscle fibres. As judged from their figures, and the few gold chloride preparation I have made of frog spindles, the general form of this primary ending i more 'flower-spray' than 'annulo-spiral'. In reptiles, birds, and mammal the primary ending is 'annulo-spiral' though its regularity varies. In mam mals its regularity in the dog (Batten, 1898) and mouse (Kerschner, 1893 approaches that found in the cat, whilst in man (Kerschner, 1893; Ruffini 1893), rat (Kerschner, 1893; Huber and de Witt, 1898), and guinea-pi (Huber and de Witt, 1898) the spiral pattern is less evident and the endin resembles the form occurring in the rabbit.

Secondary endings of the 'flower-spray' form as found in the cat have bee

observed in the hedgehog (Garven, 1925), the pig (Cuajunco, 1927), and man (Ruffini, 1893). Tello (1922) described its form as 'claw-like' in six-month human foetal material. Denny-Brown (1928*a*) found secondary endings to occur in complex spindles from the extrinsic eye-muscles of sheep, and maintained that they were identical with those found in similar spindles from the cat's gastrocnemius. Cilimbaris (1910), studying the same material, figured endings which were apparently secondary and of the 'flower-spray' type, though he made no attempt to correlate his observations with Ruffini's. The ending has not been previously explicitly recognized in its 'annulo-spiral' form, although the figures of Huber and de Witt (1898, fig. 33) and Dogiel (1902, figs. 14, II and V) clearly show spindles of the rabbit with primary and secondary endings both of the 'annulo-spiral' type. Faced by one extensive system of spirals rather than by two distinct types of termination, Dogiel concluded that Ruffini's descriptive terms applied to the various regular (annulo-spiral) and irregular (flower-spray) *portions* of the endings and considered such a classification superfluous. Similarly Huber and de Witt supposed that Ruffini's 'flower-spray' ending was merely the more irregular terminal portion of a spiral ramification. It seems highly probable that the spindles described by Huber and de Witt as 'compound', with two or three 'areas of nerve distribution', were spindles with a primary ending accompanied by one or two secondary endings. They found such spindles in the tortoise, dove, and various mammals. They illustrate a 'compound' spindle from the intrinsic plantar muscles of the dog (1898, fig. 39) which is without doubt of the complex type that occurs in the rabbit, with a secondary ending of 'annulo-spiral' form lying on each side of the primary ending.

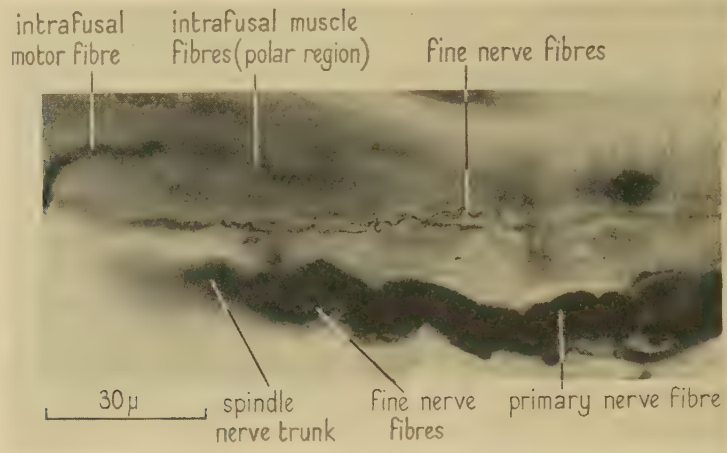
In view of the varying form of the two types of sensory termination the terms 'primary' and 'secondary' would seem preferable. These terms adequately express the facts that the primary ending is always present whilst the secondary ending only occurs in the intermediate and complex type of spindle; that the primary fibre is larger than the secondary; and that during development, according to Tello (1922), the primary fibre is the first to reach the end-organ whilst the secondary fibres arrive later together with the motor fibres.

Other Nerve-fibres innervating the Muscle-spindle

The muscle-spindle receives a rich vascular supply, and fine nerve-fibres are commonly seen accompanying the blood-vessels. In silver preparations they run a characteristically sinuous course and measure approximately 0·5 μ in diameter. There is no increase in diameter before branching, nor any constriction at the point of division. The divisions are dichotomous and the branches diverge widely apart. These fibres have previously been noted by Huber and de Witt (1898), Dogiel (1902), and Tello (1922), and were considered by these authors to be sympathetic. This was later proved correct by Hinsey (1927), who found that such fibres were the only ones to remain intact after a degeneration of the somatic innervation of the spindle, and by

Hines and Tower (1928) who found them to be the only spindle nerve-fibre which degenerated in sympathectomized material.

There are other fibres, finer than these, which appear to take part in the innervation of the spindle itself. Perroncito (1902) first noted their existence in muscle-spindles of the lizard. He observed two or three very thin fibres which approached the spindle together with the primary fibre and apparently ran within its endoneurial sheath. On reaching the equatorial region these ramified and spread out to form an anastomosing network within the walls of the capsule. Tello (1922) observed similar fibres innervating spindles of the cat and man in the later stages of development. They bore no relation to the



Text-fig. 13. Part of a polar region of a rabbit's muscle-spindle with portion of spindle nerve-trunk alongside. Very fine fibres accompany the primary nerve-fibre in the nerve trunk and also course over the polar region. M. vastus intermedius, L.S. 25 μ, de Castro silver method.

blood-vessels but ran chiefly within the layers of the capsule so as to encircle the equatorial region. Sometimes the fibres appeared to leave the spindle without terminating; alternatively they formed endings on the intrafusal muscle-fibres, though the mode of termination was obscure. Garven (1925 m. panniculus carnosus of hedgehog) describes fine unmyelinated fibres entering the spindle with the other nerve-fibres and forming a complex network of ramifications inside the capsule. He failed to find them ending upon the muscle-fibres. On the other hand, Creed *et al.* (1932) state that unmyelinated fibres, entering the spindle with the primary fibre, frequently end 'as minute rings or plates epilemmally placed among the nuclei of the sheath of the intrafusal muscle-fibre, often in the region of the annulo-spiral ending'. Cuajunco (1940, human foetal material) maintains that branches of unmyelinated fibres actually join the ramifications of the sensory ending. Since other branches appeared to innervate the walls of blood-vessels he concluded that the fibres were of sympathetic origin.

In the rabbit spindles examined these fine fibres were not a constant occurrence and could only be detected in silver preparations (see Text-fig. 13). They are so small (less than 0·5 μ) that it is impossible to be certain of the presence or absence of a myelin sheath. Sometimes one such fibre penetrates the capsule with a sensory fibre, takes a meandering circling course within the capsule walls, and proceeds into one of the polar regions. Here it divides extensively to form a fine anastomosing network. Alternatively, a small bundle of these fibres may enter the spindle at one pole together with other spindle nerve-fibres. Some of them divide so as to form a network over part of this polar region, whilst others, dividing *en route*, wind their way through the walls of the capsule and form a network in the other polar region. I have been unable to determine whether this network is confined to the connective tissue sheath surrounding the intrafusal muscle-bundle, or whether it is in contact with the muscle-fibres themselves. In the spindles examined the fibres have never been observed to end amongst the sensory terminations.

The fact that these fibres frequently approach a spindle in the form of a bundle, their course and distribution within the spindle, and their occurrence in one spindle but absence in another nearby in the same preparation, leaves no doubt that these fibres are nervous and not artifacts. Similar fibres have been described as taking part in the innervation of tendon-organs by Ruffini (1897, 'concomitant fibres') and Tello (1922, 'accessory fibres'). Dogiel (1902) also observed thin myelinated fibres forming a fine network within small types of Pacinian corpuscles. I have seen such a corpuscle, innervated as he describes, lying under an aponeurosis of m. vastus medialis of a lamb. The nature and function of these fibres is at present unknown. It is possible that they are somatic sensory fibres subserving the sensation of pain.

Discussion

It has been shown by Sherrington (1894) and Eccles and Sherrington (1930) that afferent fibres 12–20 μ in diameter are present in considerable numbers in muscular nerve-trunks. On histological grounds there is no alternative but to suppose, as Sherrington (1894) suggested, that the majority of these large fibres terminate in muscle-spindles and tendon-organs, while a certain number are presumably accounted for by Pacinian corpuscles. However, some reconciliation must be made between the fact that when the large primary fibres innervating the muscle-spindle and the fibres innervating the tendon-organs are measured in the neighbourhood of these end-organs, their diameter lies between 8 and 12 μ and rarely exceeds 12 μ, while fibres of this size are poorly represented in the muscular nerve-trunk. It is possible that larger afferent fibres subdivide into fibres of these dimensions, though the data of Eccles and Sherrington suggest that very little subdivision of afferent fibres above 10 μ occurs as the nerve-trunk approaches the muscle. Intramuscular subdivision has been observed by Golgi (1880), Cattaneo (1888), and Dogiel (1906) to occur in the case of the tendon-organ afferents, one fibre branching so as to innervate several end-organs, and a similar condition has

often been described for the innervation of Pacinian corpuscles, but the sensory fibres innervating the muscle-spindle show no sign of being derived by branching from a parent fibre when traced back a few millimetres from the spindle. The discrepancy in the diameter measurements might be due to the diameter of the fibres progressively diminishing in their course from dorsal root to end-organ. The observations of Sherrington (1894) on osmic material suggest that such diminution may occur; an examination of several post-thoracic dorsal roots revealed a number of fibres 23–5 μ in diameter, whilst in the muscular nerve-trunks the largest fibre found was 20·5 μ and fibres of 20 μ were rare, and in intramuscular nerve-trunks the fibres were rarely 'quite as large as the largest afferent fibres seen in the parent nerve-trunks traceable into the muscle by naked eye dissection' (p. 235).

Whatever the explanation of this discrepancy, the fast conduction rate of the afferent impulses initiated when a muscle is stretched supports the conclusion that the 12–20 μ group of afferent fibres in muscular nerve-trunks is proprioceptive. Recently Lloyd (1943) has applied brief stretch stimuli to the cat's gastrocnemius and recorded the resultant afferent discharge at several points along the nerve and dorsal root between the muscle and spinal cord. He showed that the afferent response was conducted at an average maximum velocity of 116 m.p.s. According to the size: velocity ratio for the cat (Hursh 1939) fibres with this conduction rate have a diameter of 18 μ. It is therefore evident that stretch receptors in the muscle are innervated by the 12–20 μ group of afferent fibres. It is impossible to determine histologically which fibres within this range ultimately become the primary (8–12 μ) and secondary (6–9 μ) afferents which enter the muscle-spindles, and the afferents (8–12 μ) which innervate the tendon-organs. The individual conduction rates of these fibres are not known, though in Matthews's (1933) single fibre preparations the time relations of the impulses recorded from the secondary endings of the muscle-spindle (A1 receptors) showed that they travelled in smaller fibres than those from the primary spindle endings and tendon-organs (A2 and B receptors). This suggests, together with Lloyd's figure of 116 m.p.s., that the larger fibres within the 12–20 μ range innervate the tendon-organs and terminate as primary endings in the muscle-spindles, and that the secondary spindle afferents are to be identified amongst the smaller fibres in this group.

Matthews (1933) concluded from his experiments that both the primary and secondary endings of the muscle-spindle responded when the muscle was stretched, but that only the primary ending was affected during contraction caused by supramaximal motor stimulation. He assumed that supramaximal stimuli excited the small motor fibres innervating the spindle and caused the intrafusal muscle-fibres to contract. The different behaviour of the two types of ending under these conditions was accounted for by supposing that whilst the non-contractile nuclear bags would become extended and so distort the primary endings during the contraction of the intrafusal muscle-fibres, the myotube regions would shorten and so decrease the distortion of the secondary endings. Impulses would therefore be initiated in one but not the other. The different

location of the two types of ending may well result in their behaving differently during intrafusal contraction, but it must be noted that the strongest motor stimulus used by Matthews was 'at least' 30 per cent. supra-maximal as judged by the height of the contraction attained by the muscle, and in view of Leksell's work (1945) it is clear that such stimuli would fail to excite the greater proportion of the small ('gamma') efferent fibres innervating the spindle. Leksell showed that a muscle attained its maximum contraction tension at a stimulus strength 2·5 times the threshold of the largest fibres in the 'alpha' group, and this agreed with the figure previously obtained by O'Leary, Bishop, and Heinbecker (1934). The whole 'alpha' group was activated when the stimulus was on an average 3·1 times that of the threshold of its most excitable fibres. The threshold of the 'gamma' efferent fibres was on an average 3·9 times the 'alpha' threshold, and a further 12-fold increase of stimulus strength was required to excite all the fibres within this group. Hence a motor stimulus approximately 30 per cent. supramaximal, such as used by Matthews, would only be sufficient to excite the larger motor fibres innervating the spindle. To ensure the maximum contraction of the intrafusal muscle-fibres a stimulus would have to be employed that was more than 500 per cent. supramaximal as judged by the height of the muscle contraction. It remains for future experiment to show whether the secondary endings would respond under these conditions.

At present the manner in which the muscle-fibres of the spindle contract is a matter for speculation, and nothing is known of the conditions of central excitation necessary for this to occur. It has been shown in this paper that in all probability each member of the muscle-bundle is a double contractile unit, the two polar portions being separated by a non-contractile nuclear bag, and each end bearing one (sometimes two) motor end-plates. It is possible that the intrafusal muscle-fibre should on the contrary be regarded as a single contractile unit, the effect of its multiple motor innervation being to ensure the simultaneous contraction of the two poles in much the same way that the possession of multiple end-plates appears to ensure an almost simultaneous contraction of the entire length of the extrafusal muscle-fibre in the frog's sartorius (see Katz and Kuffler, 1941). However, the fact that the two poles are separated by a region full of nuclei and devoid of cross-striations, and are often innervated by motor fibres differing considerably in diameter, would appear to weigh against this view. In those spindles where one polar region is innervated by small ('gamma') motor fibres and the other by a relatively large ('alpha') motor fibre, it seems probable that the contraction of the two poles will not be simultaneous. If each pole is innervated by large and small motor fibres their differing rates of conduction may result in the individual polar units contracting separately. Since each sensory fibre entering the spindle invariably ramifies so as to form endings around each muscle-fibre, the question arises as to what the nature of the impulses discharged from the entire termination would be during such progressive contraction. It is interesting to note in this connexion that in some of his recordings from primary endings

during contraction Matthews found that the change-over from silence to response was gradual, the rate of discharge increasing as the motor stimulus was increased. He suggested that this might be due to the muscle-fibres of the spindle contracting separately.

Leksell found that the conduction rate of motor fibres in the small 'gamma' group was 20–38 per cent. that of the largest 'alpha' motor fibres. Hence it is possible that the intrafusal muscle-fibres innervated by this group will still be in a state of contraction when the ordinary muscle-fibres are relaxing. It has been shown that the extreme ends of the muscle-fibres of one or both poles of a spindle often taper off and fuse with the endomysium of ordinary muscle-fibres. During the relaxation of the muscle, therefore, the elongation of the extrafusal muscle-fibres by their antagonists may apply a stretch stimulus to the contracting muscle-fibres of the spindle. Maybe it is only under these conditions that impulses are discharged by the secondary endings in the course of contraction. However, these matters can only be elucidated by studying the behaviour of the sensory endings in single fibre preparations at all gradations of motor stimulation.

The form and disposition of the sensory endings of the spindle suggest that they are stimulated by mechanical distortion. During stretch, as Matthews (1933) remarks, 'it is difficult to see how tension could stimulate except by the distortion it produces.' During contraction it would seem unnecessary to postulate any other mode of stimulation such as by the action potential of the intrafusal muscle-fibres, or by pressure exerted on the endings by the contracting extrafusal muscle-fibres via capsule and lymph space. It has been suggested by Hinsey (1927) that when the intrafusal muscle-fibres contract the nerve-endings may be stimulated by being pressed against the axial sheath which overlies them. The 'flower-spray' type of ramification which spreads over the surface of several muscle-fibres would appear to be well suited to respond to this form of stimulation, but the annular and clasping forms of ending are more probably primarily adapted for stimulation by a change in form of the muscle-fibres they entwine. At present it is difficult to ascribe any functional significance to the regularity or irregularity of a sensory spindle ending. The primary ending of the cat (Matthews, 1933) has been found to respond during stretch and contraction in a similar way to the corresponding ending in the frog (Matthews, 1931*b*), yet these endings are markedly dissimilar in form (see p. 172). In both animals the ending is associated with the nuclear bags of the intrafusal muscle-fibres which suggests that it is the position of the endings within the equatorial region rather than their form that is the important factor affecting the response. Mechanical distortion would also appear to serve as the mode of stimulation for the tendon-organ endings. This may be partly provided by stretch (Eccles, personal communication to Matthews, 1933), but the distribution of these end-organs in the deep rather than the superficial laminae of tendons (Golgi, 1880), and their occurrence within the muscle in the neighbourhood of aponeuroses and intramuscular tendons suggest that distortion by compression may also provide a mode of stimulation.

It has long been apparent that among the afferent fibres proceeding from a mammalian extensor muscle such as the quadriceps there are some which reflexly excite its contraction and others which reflexly inhibit it. Though there is every reason to suppose that the afferent fibres concerned in this excitation and inhibition are those innervating the muscle-spindles and tendon-organs, the identification of the sensory endings which fulfil these opposing functions still awaits conclusive demonstration. In attempting to account for the cessation of action currents in a muscle ('silent period') which occurs during its brief reflex contraction in response to stretch (as in knee- and ankle-jerks), Fulton and Pi-Suñer (1928) and Denny-Brown (1928*a*, *b*) were led to propose conflicting theories with regard to the reflex effects of the afferent impulses discharged by these receptors. Fulton and Pi-Suñer concluded that the impulses set up by the muscle-spindle were excitatory and that the silent period was due to the fact that these end-organs ceased to discharge when unloaded by extrafusal contraction ('in series' behaviour). They suggested that impulses initiated by the tendon-organs, which lay 'in parallel' with the contractile elements, were probably inhibitory. Denny-Brown, on the other hand, believed that the reflex effect of the tendon-organ discharge was excitatory, and supposed that efferent activity during the silent period was initially prevented by a refractory state of the motor neurones and later prevented by an inhibition arising from the sensory endings of the muscle-spindle. When Denny-Brown advanced this view the presence of an afferent discharge from the spindle during stretch was open to doubt and he formulated his hypothesis on the assumption that the sensory endings of the spindle discharged only during contraction. However, Matthews (1931*a*, *b*) was able to provide conclusive evidence that impulses were set up by the spindle both during the stretch and the contraction of muscle, and the behaviour of his single fibre preparations (1933) conformed with that proposed by the 'in series, in parallel' hypothesis. Matthews supposed that the silent period was due partly to an inhibitory discharge from the tendon-organs and partly to the absence of an excitatory discharge from the muscle-spindles. The silent period was known to be most pronounced during the contraction of muscle at low initial tensions, and Matthews's recordings showed that the tendon-organ was the only receptor which discharged during contraction under these conditions. Therefore if the latter part of the silent period was inhibitory in origin as Denny-Brown had maintained, the tendon organ alone could be held responsible for it. Matthews believed that the contraction of muscle in the stretch reflex was excited by the muscle-spindles, and suggested that their secondary endings should be regarded as those which initiated the excitatory impulses on account of their very low threshold of response to stretch. He was undecided as to the reflex nature of the discharge from the primary ending of the spindle, but inclined to regard it as inhibitory.

The spindle is now generally assumed to be the receptor which excites the stretch reflex and in this connexion is often simply referred to as the 'stretch afferent'. However, much of the evidence advanced in support of this view

is either invalid or inconclusive, and its acceptance ultimately depends upon Matthews's finding of a considerable difference in threshold between the spindle and tendon-organ in response to stretch. In his preparations a tension of 1–2 gm. was sufficient to evoke a rhythmic discharge from the secondary ending of the spindle and the primary ending discharged at tensions of 5–10 gm.; on the other hand, in the case of the tendon-organ, a tension of 20–200 gm., or in some preparations even 700 gm., was required to initiate a discharge. The low threshold of the receptor exciting the stretch reflex is indicated by the fact that stretches of a fraction of a millimetre are sufficient to elicit the contraction (see, for example, Liddell and Sherrington, 1924; Denny-Brown and Liddell, 1927; Lloyd, 1943). A correlation of this characteristic of the reflex with Matthews's threshold data therefore suggests that it is the spindle rather than the tendon-organ which excites the response. If this is so it is clear that the large primary fibre innervating the spindle should be identified as the 'stretch afferent' rather than the smaller secondary fibre specified by Matthews, for the brief latency of the reflex and rapid conduction rate of the afferent discharge exciting it (Lloyd, 1943) indicate that sensory fibres of the largest diameter are employed. The functional significance of the secondary endings, their occurrence in some spindles in mammalian muscle but not others, and their complete absence from amphibian spindles are matters to which at present there is no answer. The reflex nature of the afferent discharge from the spindle during intrafusal contraction is equally obscure. Does the spindle excite the contraction of its own muscle-fibres and in so doing initiate a further excitatory discharge from its sensory endings?

The low threshold of the spindle in response to stretch suggests that it may well be the receptor which excites the stretch reflex, but the other evidence which has been advanced in support of this view does little to strengthen it. For example, it has been argued (Fulton, 1926; Fulton and Pi-Suñer, 1928) that since the stretch reflex of a muscle is unimpaired when its tendon is cut away or anaesthetized, the tendon-organ can be excluded as being the proprioceptor which bears the excitatory afferent. The experiments of Morson and Phillips (1937) were also based on the assumption that tendonectomy ensures the removal of the majority of tendon organs. However, the tendon-organs of a muscle are found wherever the muscle and tendon fibres composing it are united. Hence they occur not only at the musculo-tendinous junctions at either end of a muscle, but also in the places where these junctions are intramuscular and under flattened expansions of tendon (aponeuroses) covering the fleshy part of a muscle. Moreover, they frequently occur in association with muscle-spindles. Therefore, as Hinsey (1927) and Denny-Brown (1928*a*) have indicated, it is impossible to achieve any mechanical separation of the two types of proprioceptor which would ensure a significant decrease in the number of tendon-organs relative to muscle-spindles. The fact that the stretch reflex is strictly confined to the portion of the muscle stretched is also cited as circumstantial evidence in favour of regarding the muscle-spindle as the end-organ which excites the response. However, the distribu-

tion of muscle-spindles and tendon-organs does not warrant this deduction; both types of proprioceptor are usually to be found in any small block of muscle taken from members of the quadriceps of the cat and rabbit.

Finally, it is urged that the muscle-spindle should be regarded as the receptor which excites the stretch reflex, since it is lacking in those muscles not subject to gravitational stretch (Fulton, 1946), and in these muscles it is commonly supposed that the stretch reflex is absent. This implies that the distribution of muscle-spindles in the body is confined to anti-gravity muscles, but it is evident from the literature (see especially Gregor, 1904) that this is not the case. Muscle-spindles occur in the diaphragm (Dogiel, 1906), in the extrinsic muscles of the larynx (Kölliker, 1889; Forster, 1894; Amersbach, 1911), and tongue (Forster, 1894; Langworthy, 1924), and are particularly abundant in muscles of fine adjustment, for example, of the hand (see, for example, Voss, 1937). Cilimbaris (1910) found a spindle in m. tensor tympani of the sheep. None of these muscles could be said to fulfil an anti-gravity function. Of the muscles in which it has been alleged that spindles are absent (intrinsic laryngeal and lingual muscles, genital, facial, and extrinsic eye-muscles) evidence about their stretch-reflex response is known only for the extrinsic muscles of the eye. This reflex could not be demonstrated in these muscles by Irvine and Ludvigh (1936) in man, nor by McCouch and Adler (1932) or McIntyre (1941) in the cat. McIntyre, moreover, failed to detect the presence of any sensory discharge during the stretch and contraction of these muscles in the cat. The reason for this is at present obscure, but these observations cannot be regarded as evidence for supposing that the muscle-spindle excites the stretch reflex since the assertion that spindles are lacking in the extrinsic eye-muscles is incorrect. They have been demonstrated to occur in their usual encapsulated multifibral form in human eye-muscles (Cooper and Daniel, 1948) and in the eye-muscles of the sheep and various mammals by Cilimbaris (1910), while they appear to be present in the unencapsulated monofibral form in the eye-muscles of the cat (Denny-Brown, 1928*a*; Pallot, 1934).

Thus the view that the 'stretch afferent' is borne by the muscle-spindle is seen to rest largely upon equivocal evidence and to receive convincing support only from the fact that it is the most probable conclusion to be drawn from Matthews's threshold data. On the other hand, it may equally well be said that there is little evidence to suggest that the excitatory receptor should on the contrary be identified as the tendon-organ. At present the only indication that this alternative view may be correct is provided by experiments in which the recovery of the stretch reflex has been observed after nerve regeneration. Recoveries have been reported by de Rezende (1942) and Davis *et al.* (1945) after nerve-grafting, and by Barker and Young (1947) after nerve-suture. In the latter investigation the reflex (knee-jerk) was found to recover to the extent of developing over half its pre-operative tension in some cases. After the complete division of a nerve one would suppose that a tendon-organ, innervated by one sensory fibre, would stand a greater chance of becoming successfully reinnervated than a muscle-spindle, which receives both a motor and a

sensory innervation. Considerable recoveries of the stretch reflex after such operations therefore suggest that the afferent fibre which excites the response belongs to the tendon-organ rather than the muscle-spindle. It remains to be seen whether a full investigation of the muscles reinnervated in Barker and Young's experiments will justify these conclusions. A preliminary examination of spindles reinnervated after nerve-suture suggests that it is very doubtful whether the motor and sensory innervation is ever fully restored.

It seems probable that all myotatic phenomena are the outcome of a fluctuating balance between two types of afferent discharge arising from muscles which have opposing reflex effects, one exciting and the other inhibiting contraction somewhat on the lines suggested by Denny-Brown (1928*a*, *b*) and Creed *et al.* (1932). Judging from the balance of present evidence it seems probable that the afferent discharge from the spindle is excitatory and that from the tendon-organ inhibitory to the motor neurones of the same muscle. However, the specific reflex functions of the sensory fibres innervating these receptors, and the manner in which the impulses they carry interact during the stretch and contraction of muscle, clearly require further elucidation.

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Summary

A study of the morphology and innervation of muscle-spindles from the quadriceps of the rabbit and cat has shown that:

1. The intrafusal muscle-fibres do not subdivide in their course through the spindle, as is maintained in some descriptions, but retain their individuality from pole to pole.

2. There is no constant feature which is characteristic of one pole of a spindle and not the other. A distinction can be made between the proximal and distal ends only when it is possible to orientate the spindle according to the proximal and distal ends of the muscle. The extreme ends of the spindle are attached indifferently to extrafusal endomysium, tendon, or perimysial connective tissue.

3. In the equatorial region each muscle-fibre of the spindle contains a dense aggregation of spherical central nuclei ('nuclear bag'). On either side of this aggregation oval nuclei are disposed in the form of a chain within a central core of protoplasm ('myotube region'). The nuclear bag is devoid of cross-striations and presumably non-contractile. The two polar portions of the muscle-fibre on either side of the bag are striated and each receives a motor innervation; hence they are presumed to function as independent contractile units.

4. The number of end-plates possessed by a spindle is approximately double its number of intrafusal muscle-fibres, with half the total number of end-plates situated at each pole. The ratio is rarely exact, since one polar half of an intrafusal fibre frequently bears two end-plates; these are innervated by nerve-fibres which retain their individuality as far as they can be traced back from the spindle. Both small nerve-fibres (3–4 μ in gold chloride preparations) and relatively large nerve-fibres (6–7 μ in gold chloride preparations) take part in the motor innervation of muscle-spindles, as was deduced on physiological grounds by Leksell (1945).

5. An analysis of the sensory innervation has confirmed many of Ruffini's (1898) observations. Primary or 'annulo-spiral' and secondary or 'flower-spray' endings occur and they are innervated by independent nerve-fibres; it is suggested that Ruffini's terms 'primary' and 'secondary' be adopted since the descriptive terms cannot always be applied. In the rabbit the secondary ending is 'annulo-spiral' in form and differs little from the primary ending; in the cat it is more irregular and could be termed 'flower-spray'. The primary ending is always present and is associated with the nuclear bags of the intrafusal muscle-fibres; in some instances its ramifications are more extensive and also entwine the myotube regions. The primary ending may be the only sensory termination present, or it may be accompanied by one or by two secondary endings. These are borne by the myotube regions of the muscle-fibres. In the rabbit's quadriceps and interossei, spindles with one primary and one secondary ending were the most frequent in the samples taken; in the cat's quadriceps spindles with one primary and two secondary endings were the most numerous. Both the primary and secondary nerve-fibres invariably ramify so as to innervate each intrafusal fibre in the muscle-bundle. The two sensory terminations are often closely intercalated but do not overlap with one another to any great extent. As estimated from measurements made on fresh, silver, and gold chloride preparations the total diameter of the primary fibre lies between 8 and 12 μ, that of the secondary fibre between 6 and 9 μ.

6. Apart from small sympathetic fibres innervating the vascular supply of the spindle, other finer fibres may occasionally be seen ramifying within the walls of the capsule and over the polar regions. It is possible that they are somatic sensory fibres subserving the sensation of pain.

7. The nature of the reflex effects of the afferent impulses discharged by the muscle-spindle and tendon-organ is considered, and it is concluded that the balance of evidence indicates that the afferent discharge from the spindle is excitatory and that from the tendon-organ inhibitory to the motor neurones of the same muscle. However, the identification of the spindle as the receptor which excites the stretch reflex is found to rest largely upon equivocal evidence, its acceptance depending ultimately upon Matthews's finding (1933) of a considerable difference in threshold between the spindle and tendon-organ in response to stretch. It is suggested that the large primary fibre innervating the spindle should be identified as the 'stretch afferent' rather than the smaller secondary fibre specified by Matthews, for the rapid con-

duction rate of the afferent discharge exciting the stretch reflex (Lloyd, 1943) indicates that sensory fibres of the largest diameter are employed. The functional significance of the secondary fibres is obscure and the specific reflex functions of the sensory fibres innervating both the spindle and the tendon organ clearly require further elucidation.

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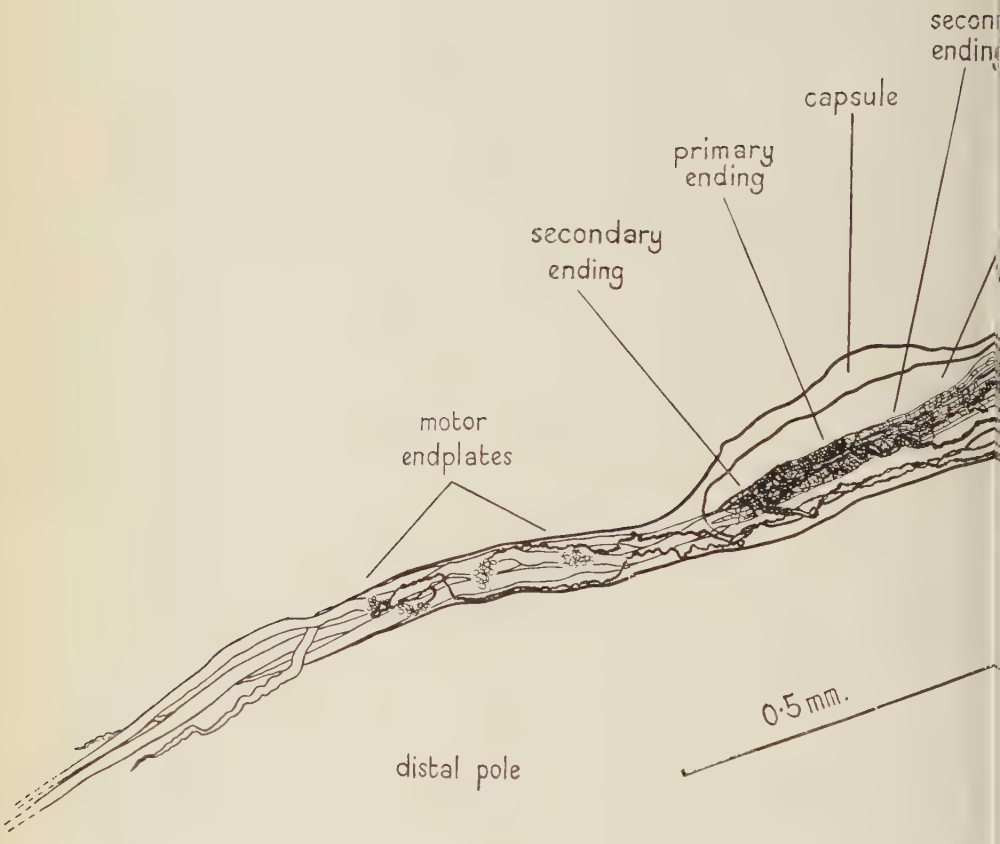
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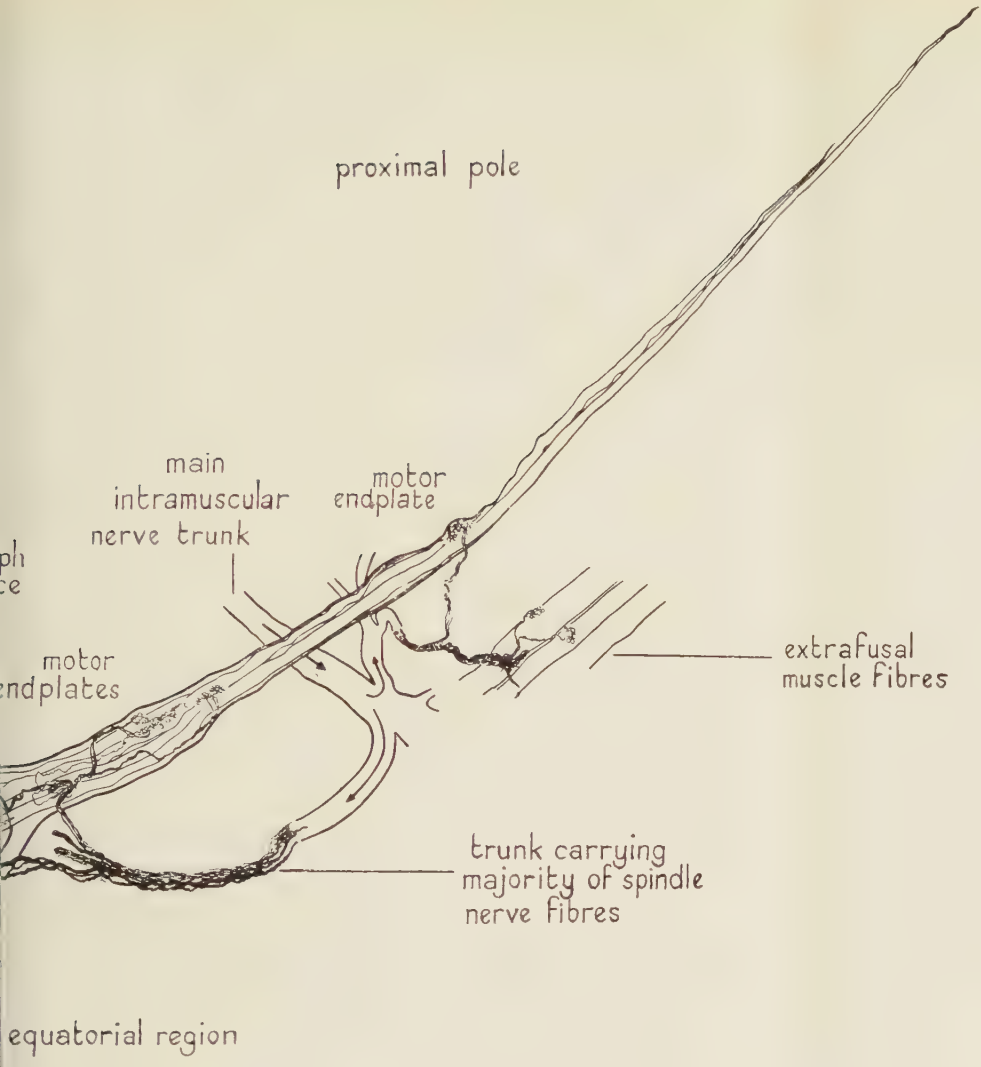
D. BARK


proximal pole
main
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nerve trunk
motor
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motor
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extrafusal
muscle fibres
trunk carrying
majority of spindle
nerve fibres
equatorial region

PLATE I

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DESCRIPTION OF PLATES

Plate I

A complex muscle-spindle of a rabbit from m. vastus intermedius reconstructed as described in the text. The figure has been reduced nine times from the original, which was over 8 feet long. Many features have been omitted (e.g. cross-striations, vascular supply, all nuclei other than those of motor end-plates and the central nuclei of the myotube regions and nuclear bags of the intrafusal muscle-fibres) in order to demonstrate the innervation to the best advantage. The extreme end of the proximal pole was attached to perimysial connective tissue; at the distal pole the two muscle-fibres whose ends could be traced were attached to the endomysium of extrafusal muscle-fibres. The spindle is fully described in the text and various portions of it are illustrated in more detail in Text-figs. 3–5, 8, 10–12; the object of this plate is merely to give a general view of the entire end-organ.

Plates II and III: Key to Lettering

ax.sh.nuc axial sheath nucleus; *b.v* blood-vessel; *br.pr.n.f* branches of primary nerve-fibre; *br.sec.n.f* branches of secondary nerve-fibre; *caps* capsule; *constr* intrafusal muscle-fibre constricted by nerve-ending; *extraf.m.e.p* extrafusal motor end-plate; *f.s* flower-spray type of ramification; *im.n.t* intramuscular nerve-trunk; *intraf.m.f(s)* intrafusal motor fibre(s); *l.sp* lymph space; *myt.rg* myotube region of intrafusal muscle-fibre; *myt.rg.nuc* central nuclei in myotube region of intrafusal muscle-fibre; *nuc.bag* nuclear bag; *p.pr.e* portions of primary ending; *plr.rg* polar region; *pr.e* primary ending; *pr.n.f* primary nerve-fibre; *sec.e* secondary ending; *sec.n.f* secondary nerve-fibre; *trab.conn.tiss* trabecular connective tissue.

Plate II

Gold chloride preparations, Gairns's method.

Fig. 1. *Cat.* Complex muscle-spindle with one primary and two secondary sensory endings from m. vastus medialis.

Fig. 2. Primary ending of spindle in fig. 1. Part of the secondary ending which lies to the left of the primary is also shown.

Fig. 3. Secondary ending which lies to the right of the primary in the spindle shown in fig. 1.

Fig. 4. *Rabbit.* Intermediate type of spindle with one primary and one secondary sensory ending from m. vastus intermedius.

Fig. 5. The primary and secondary endings of the spindle in fig. 4.

Plate III

Silver preparations of spindles from m. vastus intermedius of rabbit; fig. 10 L.S. 20 μ, Holmes's method, the remainder L.S. 25 μ de Castro's method.

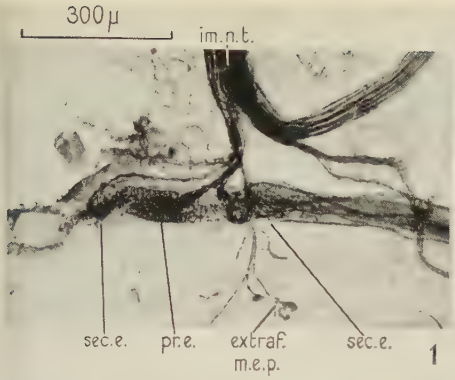
Fig. 6. Nuclear bags of intrafusal muscle-fibres and portions of primary ending. In places the ramifications can be seen constricting the intrafusal muscle-fibres; they are closely applied to the 'sarcoplasmatic membrane' and lie underneath the sarcolemmal and axial sheaths.

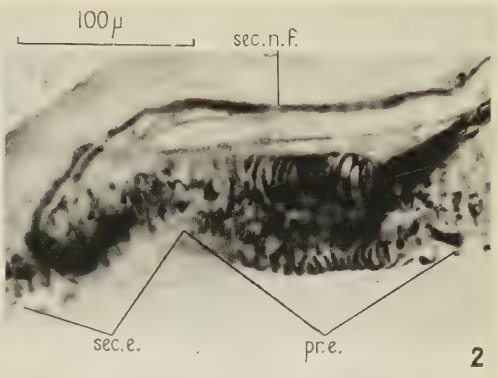
Fig. 7. The spiral complex *s* of the secondary ending in Text-fig. 12 illustrated by optical sectioning; A lowest, E highest plane of focus. The two intrafusal muscle-fibres are kinked at one point.

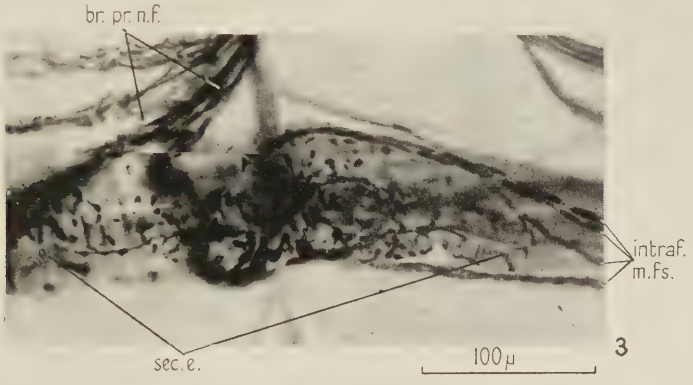
Fig. 8. Part of a secondary ending showing two complete rings amongst the ramifications.

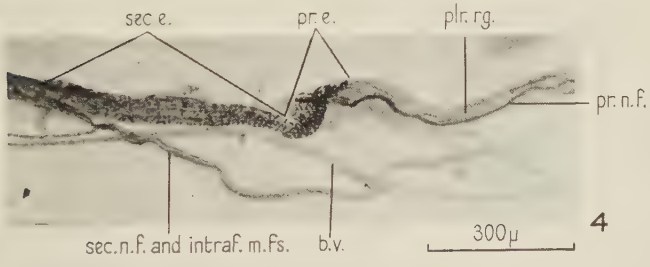
Fig. 9. Section showing the major portion of another secondary ending. Portions of the neighbouring primary ending lie to the left of the figure.

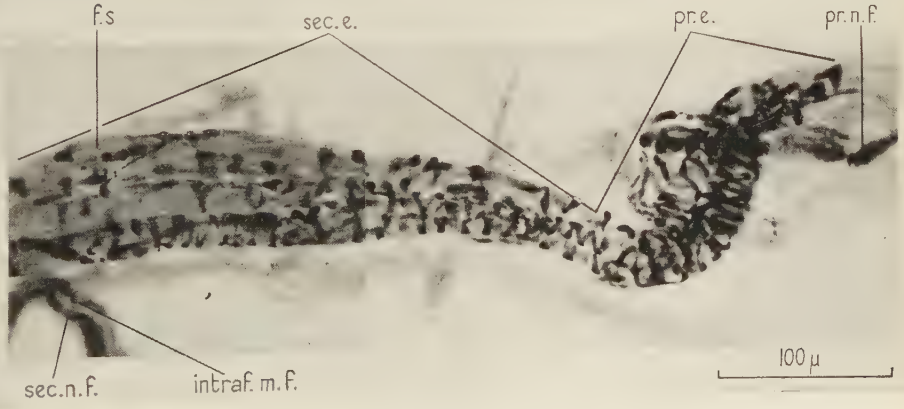
Fig. 10. Part of a primary ending; the turns of a loosely coiling spiral entwine one end of a nuclear bag and part of the myotube region of an intrafusal muscle-fibre, the typical location of this type of ramification. The nuclear bags lie to the right of the figure and the myotube regions to the left in the portions of the intrafusal muscle-fibres included in the section.



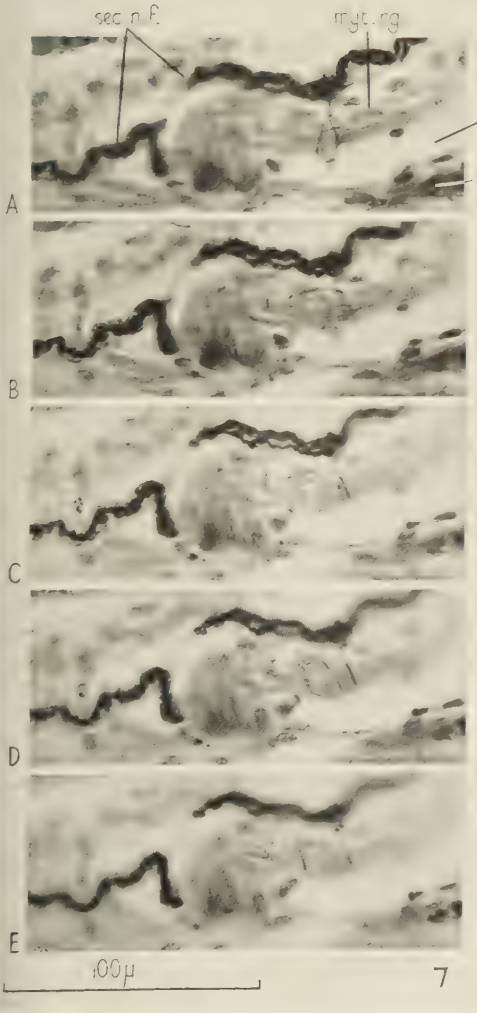


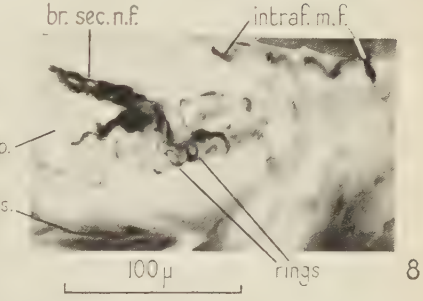


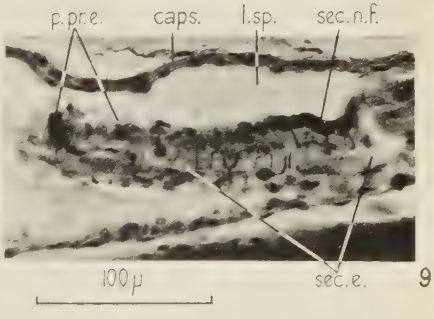


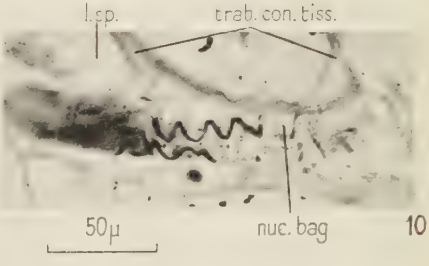


D. BARKER—PLATE II









D. BARKER—PLATE III

The Cultivation of Adult Mammalian Skin Epithelium *in vitro*

BY

P. B. MEDAWAR

(*From the Department of Zoology, University of Birmingham*)

With five Text-figures

THE purpose of this paper is to describe a simple technique for the short-term cultivation of adult mammalian skin epithelium *in vitro*. The conditions of cultivation are such as allow the rapid proliferation and migratory spread of skin epithelium while maintaining the characteristic functional activity and histological appearance of its cells.

The technique was devised to investigate the effect of iso-antibodies on cells. It requires little, if any, modification for use as an adjunct to routine biochemistry (e.g. in studying the influence of specific metabolic inhibitors on cell movement and division: cf. Medawar, 1947), or for submitting cells to the direct action of hormones. It has the disadvantage that the cultivated cells do not grow away from the explanted tissue into the culture medium, so that the direct microscopic study of living cells (an amenity to which, in conventional tissue culture, most others have been sacrificed) must be forgone. The tissue can, however, be studied in the transformed state in which it is most familiar to histologists: in stained sections.

Methods

One central principle of adult tissue culture was discovered by Parker (1936, 1937): many adult tissues require more oxygen than their embryonic precursors to initiate and maintain growth and functional activity. A second innovation of Parker's, likewise adopted here, was the culture of adult tissues for functional survival in fluid media. The long latent period that precedes the outgrowth of cells from adult tissue when it is embedded (or, as it may prove, embalmed) in a plasma jelly surrounded by air probably represents the time taken for adult cells to adjust themselves to growth at oxygen tensions lower than they have been accustomed to. Some adult tissues—spleen, for one—do not make this adjustment and usually die. On the other hand, it is shown here that adult skin epithelium will migrate vigorously and proliferate moderately at an oxygen tension no higher than that provided by a serous culture medium kept in gaseous equilibrium with air. At higher tensions, cell division quickens up and migratory activity is relatively subdued.

Principle of the Method. Thin, lightly vaselined, skin slices are floated raw side down in a stoppered test-tube or flask on 3–20 ml. serum kept in gaseous

equilibrium at 38° C. with air or an air-oxygen mixture by a smooth rotating or rocking motion. Cultures in high-oxygen media may be maintained up to 8 days, but preferably not beyond 6 days, without interference. By then the dermis may have become completely enclosed in epidermal epithelium that has migrated around it, the graft being sealed off by a layer of cuticle of its own formation. Cultivation beyond eight days would certainly require sub-culture by division and reopening of the encysted explant. Although there is no reason (except the increasing danger of infection) to doubt that it could be achieved, the prolonged cultivation of skin epithelium, being unnecessary for the purpose in hand as for many others, is not reported on here. Skin explants in a gas phase of air, in which migration is more vigorous, usually achieve complete self-encystment in 4 days, and the epithelium farthest from the free surface tends to show ischaemic changes thereafter.

Skin and culture media from adult rabbits have been used throughout. The culture fluid need not be taken from the skin donor. Explanted skin will indeed grow with undiminished vigour in the presence of body fluids, tissues, and tissue extracts from rabbits heavily and specifically immunized against it (Medawar, 1948).

Preparation of Skin for Culturing. Strict cleanliness can be achieved but asepsis can only be aspired to: the method of skin preparation described in full by Medawar (1947) has been adhered to throughout. The most convenient size for explants is a 3×4 mm. rectangle or a 3×3 mm. square. The thinner they are the better; but, although routine work has been confined to the use of explants only 0·5 mm. thickness, minute 'pinch grafts' as thick as $1\frac{1}{2}$ mm. centrally and tapering down to epidermal thickness at the edge have been used with success.

Between removal and explantation it is convenient to lay the skin fragments raw side down in a Petri dish containing a filter paper damped with Ringer's solution.

The Culture Medium. Serum has been used as the basis of the culture medium in the majority of tests; either plain, or as an extractive for minced adult tissues. Krebs-Ringer-bicarbonate containing adult tissue extract, or defibrinated whole blood diluted with four times its volume of clear serum, have both been found to support 4 days' vigorous growth in a gas phase of air.

Serum is most conveniently prepared by spinning down spontaneously clotted whole blood which has been received directly into a centrifuge tube from the median ear artery in dilatation (Medawar, 1946). Alternatively, the blood can be defibrinated immediately upon removal by twirling in it a fine glass rod with a ∩-shaped ending.

Whatever the chosen medium, eight volumes have as a routine measure been diluted with 1 vol. 5 per cent. glucose in H_2O and 1 vol. streptomycin solution, 200 u.ml. in Ringer. Streptomycin and glucose solutions were sterilized by passage through a no. 5 porosity sintered glass filter and stored in sealed 1-ml. ampoules at room temperature awaiting use. Before using

streptomycin as an internal antiseptic, on the recommendation of Dr. E. S. Duthie, approximately 50 per cent. of all cultures of 4 days' standing were lost by infection. No 4-day culture has been lost since its adoption, and only some 20 per cent. of cultures prolonged to 8 days in a single vessel. Strictly controlled tests have failed to reveal that streptomycin has any harmful effect on cells at a final concentration of 20 u./ml. in the culture medium, and concentrations as high as 100 u./ml. have been used.

It goes without saying that all equipment has been sterilized and all tissues have been handled with the precautions necessary for culture work.

Since the skin fragments are floated on the surface of a culture medium kept constantly in motion, the volume of the medium and its depth are less important than they would be with the use of submerged tissues in stationary media. By using large volumes of culture fluid, the special precautions taken by Parker (1936, 1937) to maintain a 'physiological' pH were found to be unnecessary. (Parker cultivated up to 100 mg. spleen tissue in as little as 2 ml. fluid, and kept the pH of the medium within reasonable bounds by using an excess of $NaHCO_3$ in the culture fluid in combination with a controlled excess of CO_2 in a periodically refreshed gas phase.) In our present experiments 11 independent glass-electrode determinations of the final pH of 4-day rocker flask cultures in a high O_2 gas phase gave a mean of pH $7{\cdot}37 \pm 0{\cdot}08$ standard error. Six independent determinations of the final pH of similar cultures of 8 days' standing gave a mean of pH $7{\cdot}21 \pm 0{\cdot}10$.

Cultivation in a Gas Phase of Air. This technique has been found suitable for skin, for epithelium from the kidney cortex, and for the duct elements (not the 'parenchyma') of liver and submaxillary gland. It is not suitable for lymph node or spleen. The behaviour *in vitro* of tissues other than skin will be described by Medawar (1948).

In all experiments using a gas phase of air (hereafter 'air cultures' for short), skin slices have been floated two or three at a time on 3 ml. culture fluid in 16×130 mm. Pyrex glass test-tubes with B-19 size standard ground-glass stoppers sealed down with stopcock grease and held firmly in place by elastic bands (Text-fig. 1*a*). In the apparatus illustrated by Text-fig. 1*b*, up to eight such tubes may be rotated simultaneously at a tilt of 15° from the horizontal in a water-bath at 38° C. Save that it is adapted for use in a water-bath, as short-period culture work requires, the apparatus illustrated differs only in details of design from the standard 'roller-tube' instrument introduced into tissue culture by Gey (1933).

The tubes should be given a sharp tap if the skin slices show a tendency to become beached on the side wall of the test-tube during the early stages of rotation. Sometimes the fragment is beached during part of its circuit and is picked up and floated during the remainder. This has proved to be unobjectionable.

Two or more skin fragments may often coalesce with each other marginally during cultivation, without appreciably impairing the growth of either one. For some purposes (cf. Medawar, 1948) it may be desirable to study the effect of one tissue on another by glueing the two together before cultivation. The

following procedure has been uniformly successful. Withdraw blood from the median ear artery and at once mix 4 ml. with 0·4 ml. 4 per cent. Na citrate. Spin down the corpuscles and decant the plasma. Mix a few drops of the plasma with one-tenth of its volume (or, if need be, more) of a 1 per cent. aqueous solution of anhydrous $CaCl_2$. Soak both tissue-pieces for a few

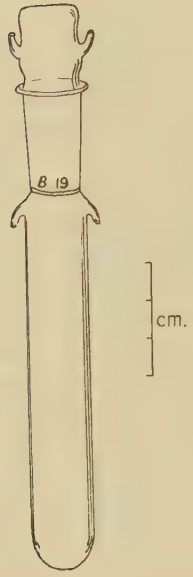


Fig. 1*a*

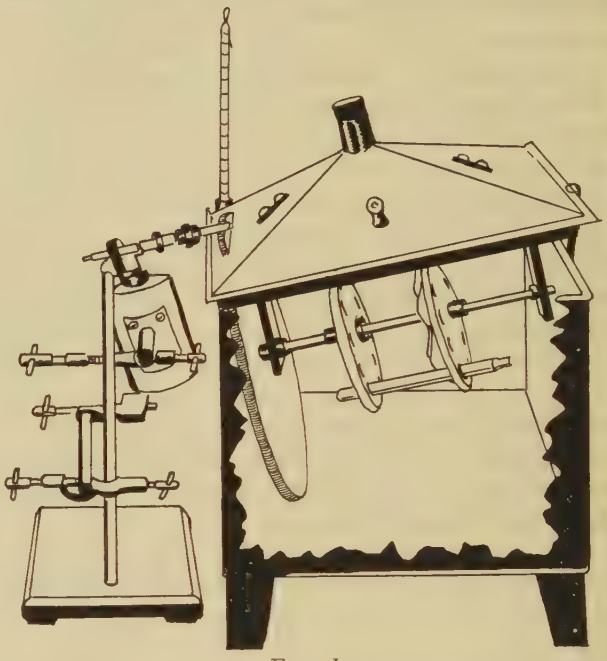


Fig. 1*b*

Text-fig. 1. Apparatus for 'roller-tube' culture: (*a*) the culture tube, and (*b*) the rotating apparatus. The rotating unit is attached to the lid of a standard bacteriological water-bath: during running it is fixed by a spring strut at a tilt of 15° from the horizontal. A stout rubber washer attached to one of the two perforated plates prevents the tubes slipping in the tilted position. It will be noted that the long axis of each culture tube remains parallel to and equidistant from the central shaft during rotation. The drive is external, and connects by a rubber bush to the large milled driving-wheel which protrudes through the lid. A resistance in series with the motor is used to maintain rotation at about 6 times per minute.

seconds in the mixture, lay them together in the disposition required, and allow about a minute for clotting to take place.

Cultivation in a High-oxygen Gas Phase. Cultures of this type are most conveniently done in conical flasks fitted with accessories for gas perfusion. Two sizes of flask have been used: of 50-ml. capacity, for fluid volumes up to 4 ml., and of 250-ml. capacity (Text-fig. 2*a*) for volumes up to 20 ml. The larger flask differs from the smaller only by having a stoppered side-tube to facilitate the introduction and removal of culture media and tissues. Being designed for periods of culture in relation to which 'warming-up time' would be negligible, the flasks were mounted in a dry-air incubator, upon a platform smoothly rocking 15° from the horizontal either way about four times per minute (Text-fig. 2*b*).

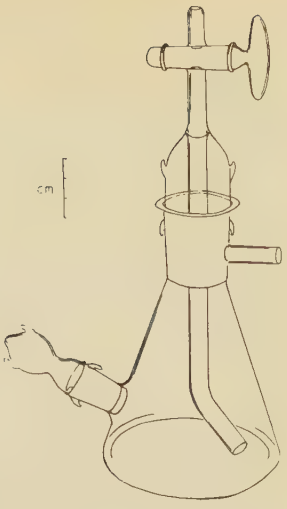


FIG. 2*a*

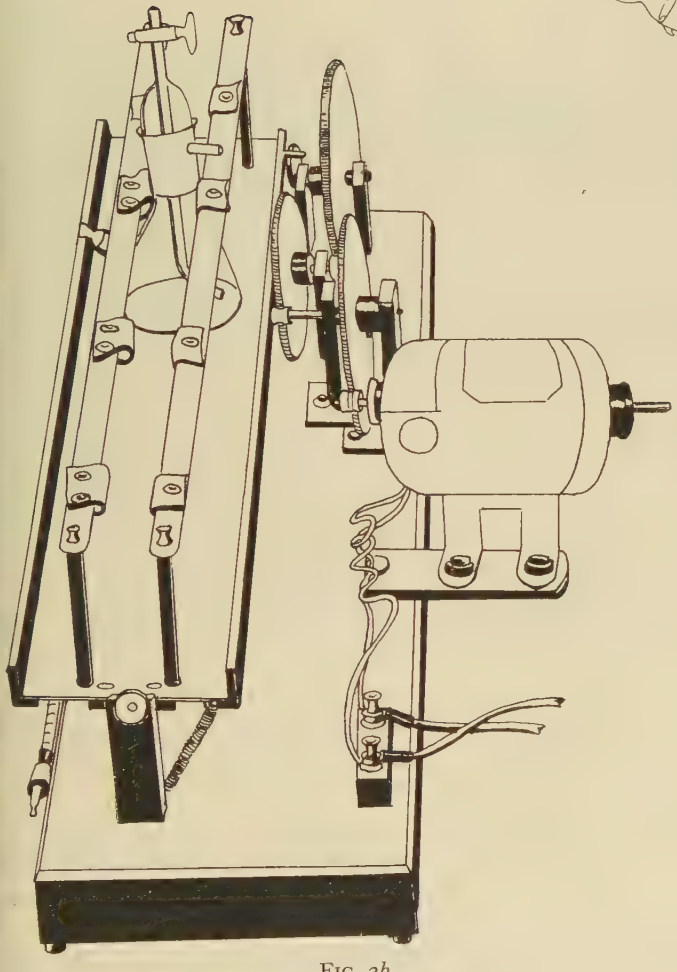
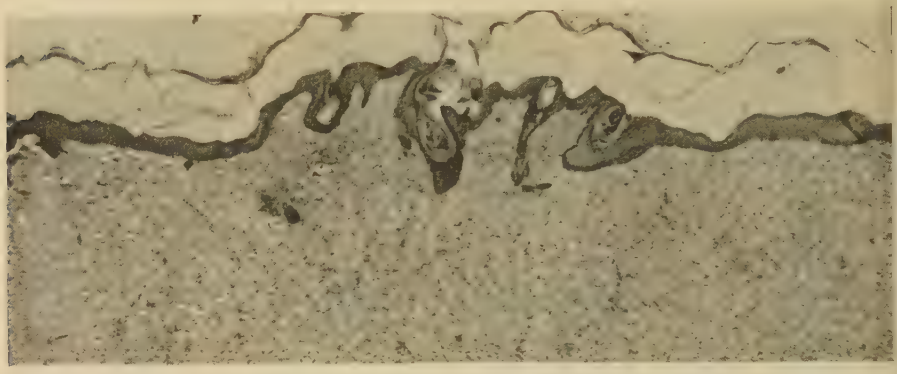


FIG. 2*b*

TEXT-FIG. 2. Apparatus for 'rocker-flask' culture: (*a*) the 250-ml. culture flask, and (*b*) the rocking device. The gas exit-tube on the neck of the culture flask is opened and closed by rotating the flask head. A small motor, suitably geared down to a cam, rocks the spring-loaded platform 15° from the horizontal either way about its long axis.

Oxygen being denser than air, the gas entry tube was prolonged towards the bottom of the flask. The exit tube, closed by rotation of the flask head, was connected to an ordinary water-displacement gas volume meter. In the writer's own experiments the exact oxygen tension of the cultivation fluid was of no importance, and sufficient uniformity was achieved by passing through the flask one measured volume of cylinder O_2 at the timed rate of 100 ml. per minute, giving a final concentration of 65–70 per cent. It is clear that if oxygen-nitrogen-CO_2 mixtures of known composition are to be introduced, at least five volumes should be passed through the flask to flush out residual air.



TEXT-FIG. 3. Illustrating the outcome of a 'survival test'. The skin culture, after 4 days *in vitro*, was transplanted back to a raw area on the chest of its donor, and removed 10 days later. The original explant is represented by the raised-up central portion of the section: its continued survival is proved by the widespread outgrowth of strongly hyperplastic epithelium from it. Ehrlich's haematoxylin and eosin; × 27.

Survival Tests; Histological Methods. When skin has been cultured in a medium suspected to affect its growth adversely, it has been found informative and reassuring to duplicate each skin slice, one being reserved at the end of the experiment for histological examination, and the other subjected to a routine test of its continued survival. Any histological method appropriate to skin can be used on skin cultures, but to avoid messiness the serum still sticking to the explants on their removal should be rinsed away with Ringer's solution before fixation.

The principle of the routine survival test is to graft the cultured skin back to a raw area on the animal from which it originally came. Outgrowth in that position constitutes an absolute proof of continued survival; necrosis indicates with slightly less complete certainty that the fragment was in fact dead. The technique has been described in full by Medawar (1947). Since a surviving explant may well have encysted itself completely, it is essential that the delicate epithelial jacket over the dermal surface should be scraped away before grafting. The unmistakable outcome of a positive survival test 10 days after grafting is illustrated by Text-fig. 3.

Results

Cell migration is faster in air than in high-O_2 cultures, and complete self-encystment (Text-fig. 4 *a*, *b*) occurs correspondingly sooner and more often. By 24 hours the shoulders of the culture are rounded and smoothed off by the

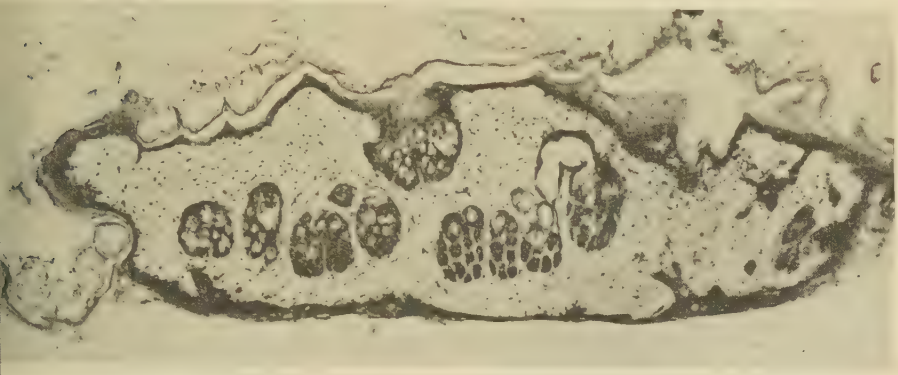


Fig. 4*a*

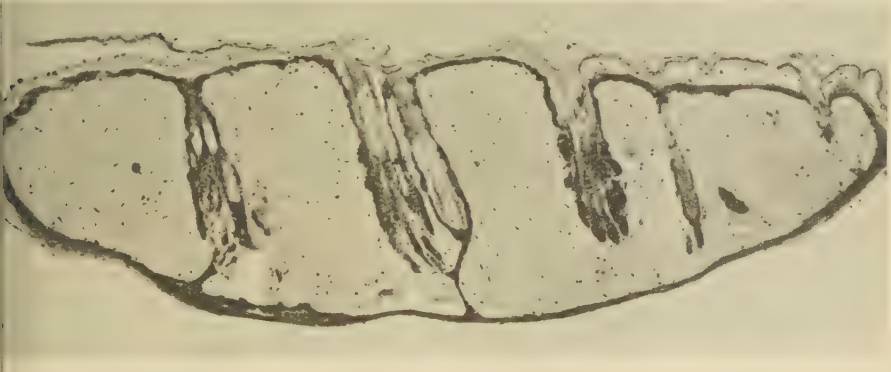


Fig. 4*b*

Text-fig. 4. Illustrating the complete self-encystment by migratory overgrowth of skin squares cultivated for 4 days in roller-tubes (gas phase: air) in serum (*b*) and serum with tissue extract (*a*). The survival of dermal mesenchyme cells is apparent in (*a*). In both the process of self-encystment has been helped by the breaking through of follicle epithelium on to the dermal surface. (The culture media were in both cases derived from a rabbit specifically immunized against the skin explanted from the donor: cf. Medawar, 1948.) Ehrlich's haematoxylin and eosin; × 47.

beginnings of migratory overgrowth, and complete self-encystment, at first always by a delicate monolayer of epithelial cells, may be achieved as early as the third day. The 'wave front' of the epithelial sheet thus advances at a rate of the order of 1 mm. per day. Migratory overgrowth may be surreptitiously helped by follicle epithelium breaking through on the dermal side of the skin piece. Though at first only a monolayer (Text-fig. 5), migratory epithelium

becomes in due course stratified, and the process of stratification occurs earliest in the regions over which migration occurred first. The epithelium is squamous, but, as with hyperplastic skin, the cuticle stays recognizably cellular and is not reduced to mere flakes.

Growth in plain serum has been found to be uniformly inferior to growth in serum which has been used as an extractive for adult spleen, lymph nodes, kidney tissue, or leucocytes.

Text-fig. 5. Illustrating the strong epidermal proliferation and relatively subdued migratory activity of a skin square rocked for 4 days in 10 ml. serum containing lymph-node fragments in a 'high-O_2' gas phase. A cell monolayer of epithelium has crept from the left of the two follicles and is beginning to grow over the dermal surface. Survival of dermal mesenchyme cells is evident. (As in the cultures illustrated by Text-fig. 4, the serum and lymph-node fragments were taken from a rabbit specifically immunized against the skin explanted from the donor.) Ehrlich's haematoxylin and eosin; × 88.

Epiboly is not the only evidence of cell migration, for, as with skin grafts *in vivo* (Medawar, 1944), there is an upward migration of follicle epithelium to the skin surface which opens out the follicle necks and eventually results in the throwing off of the hairs carried over with the explant. In fact, with 'overgrowth' deputizing for 'outgrowth', a skin explant *in vitro* behaves just like a skin graft transplanted to a raw area large enough to permit outward spread of epithelium from the graft centre. Correspondingly, the histological appearance of explanted skin epithelium is hardly distinguishable from that of ordinary hyperplastic skin.

Cell division in air cultures occurs in all regions of the epithelium, including the newly formed, except at its extreme lip. An extensive series of mitotic counts (Medawar, 1948) has shown that it is slightly more frequent at the

thickened shoulders of the culture than elsewhere. The mean frequency to be expected in an air culture when fixed after 4 days' residence *in vitro* is of the neighbourhood of one mitotic figure per mm. of superficial epidermis per 8 μ section.

Epiboly, and migratory activity in general, is less vigorous in high O_2-cultures than in those run in air. Cell division, on the other hand, is more rapid, so that the superficial epithelium comes to acquire the deep stratification characteristic of strongly hyperplastic skin (Text-fig. 5). The continued formation of normal cuticle and the persistence of sebaceous gland epithelium (Text-fig. 5) is evidence that the characteristic functional activity of skin epithelium has not been impaired.

Besides epithelial elements, fibroblasts, monocytes, and vascular endothelium survive and proliferate in cultured skin, whether in a high-oxygen gas phase or in air. The collagen fibres retain their normal packing better in air cultures than in high-oxygen cultures, possibly because in the former they are more rapidly protected by the overgrowth of an epithelial sheath. In high-oxygen cultures of 6 or 8 days' standing the fibres sometimes swell and become slightly waterlogged in appearance (Text-fig. 5); and this is associated with a tendency for the epidermis to part from the dermis in odd patches where the dermal papillae are 'oedematous'.

Discussion

The fine opportunities that the technique of tissue culture offers for the study of living cells under the microscope has caused it to develop along somewhat narrow lines. The study of cultured cells under the microscope requires not merely growth but *out*growth. Outgrowth is easier to achieve by cultivation in blood-plasma jellies than otherwise. Embryonic tissues, with their relatively low demands on oxygen, grow far better in plasma jellies than their adult successors. The association of tissue culture with the growth of embryonic tissues in plasma, or at least with growth in plasma, has therefore come to be regarded as little short of obligatory. Alternatively, it has come to be widely believed that growth 'does not occur' in fluid media, or that the growth of adult tissues, being slow of inception and dilatory in progress, renders them unsuitable for systematic use.

The experiments described here combine with those of Parker (1936, 1937) to show that the rapid organized growth of adult tissues in fluid media is so easy to achieve as to put the method within reach of any laboratory dealing with problems for which tissue culture technique might offer hope of solution. Since virtually unlimited quantities of adult tissue can be cultivated with no more difficulty than is entailed by the provision of flasks large enough to hold them, it is to be hoped that the metabolism of growing and moving cells will come to be studied by more direct methods than hitherto.

The cost of the experimental animals used in this investigation was met by the Medical Research Council; of the special apparatus, by the Department of

Plastic Surgery, Oxford University. The photographs were taken by Mr. D. A. Kempson, and the drawings made by Miss Jean Morpeth.

SUMMARY

Adult skin epithelium migrates and proliferates strongly when incubated at body temperature in a stirred serous fluid medium in gaseous equilibrium with air or an air-oxygen mixture.

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The Structure and Deposition of the Cuticle in the Adult Mealworm, *Tenebrio molitor* L. (Coleoptera)

BY

V. B. WIGGLESWORTH

(*Agricultural Research Council Unit of Insect Physiology, Department of Zoology, Cambridge*)

With two Plates and eight Text-figures

IN three earlier papers (Wigglesworth, 1933, 1945, 1947*a*) the structure and deposition of the cuticle of *Rhodnius prolixus* have been described. The main conclusions reached were as follows. The epicuticle is a composite structure made up of four layers: (i) a 'cuticulin' layer composed of a condensed lipoprotein subsequently tanned by quinones; (ii) a layer of material rich in dihydroxyphenols; (iii) a thin layer of crystalline, orientated wax molecules responsible for the waterproofing of the cuticle (cf. Beament, 1945); and (iv) an outermost 'cement' layer protecting the wax.

The lipoproteins which form the foundation of the epicuticle are apparently synthesized by the oenocytes before being transferred to the epidermal cells. The oenocytes reach their maximum development immediately before the cuticulin layer is deposited and then diminish rapidly in size. The polyphenol layer is secreted from the epidermal cells by way of the pore canals which appear to penetrate the cuticulin layer. The wax is secreted in the same way immediately before moulting. The cement layer is poured out from the dermal glands over the surface of the wax immediately after moulting.

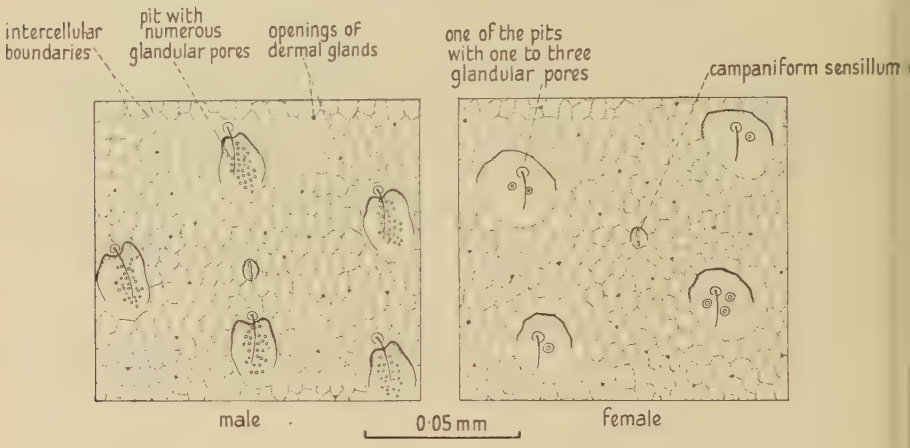
The hardening which takes place shortly after moulting is due to the tanning of the lipoprotein of the epicuticle and the cuticular protein of the exocuticle by quinones produced by the oxidation of the dihydroxyphenols (Pryor, 1940*b*; Pryor, Russell and Todd, 1947). The endocuticle is laid down by the epidermal cells during the next few days.

In the deposition of the cuticle the epidermal cells first separate from the old cuticle and undergo active mitosis. Many more cells are produced than are required. Consequently, great numbers of them suffer autolysis with the formation of the 'chromatic droplets', until, finally, a regular epithelium with one nuclei evenly spaced is produced, which then proceeds to lay down the new cuticle (Wigglesworth, 1943*a*).

If the cuticle of *Rhodnius* (and many other insects) is gently rubbed with alumina dust, the cement layer and the wax layer are abraded; the layer containing the polyphenols is then exposed and will stain a deep chestnut-brown if the insect is immersed in ammoniacal silver hydroxide. The polyphenol layer is likewise exposed more or less readily by extraction of the cuticle surface with chloroform and other wax solvents.

The ease with which the polyphenols are exposed by these two procedures varies greatly in different insects and in different parts of the same insect. In adult beetles it was found that there is no detectable abrasion (or, at least, no exposure of silver-reducing materials) over the hard regions of the cuticle in insects left in contact with alumina dust (Wigglesworth, 1947*b*). Silver staining after this treatment is confined to the soft dorsal cuticle of the abdomen and to the various intersegmental membranes.

The object of the present work was to compare the structure and formation of the cuticle in these different regions of the body in the adult beetle *Tene-*



Text-fig. 1. Surface view of ventral abdominal cuticle of *Tenebrio* adult.

brio, and to see how far the conclusions arrived at from the study of the *Rhodnius* cuticle were applicable to this unrelated insect.

Structure of the Adult Cuticle and Epidermis

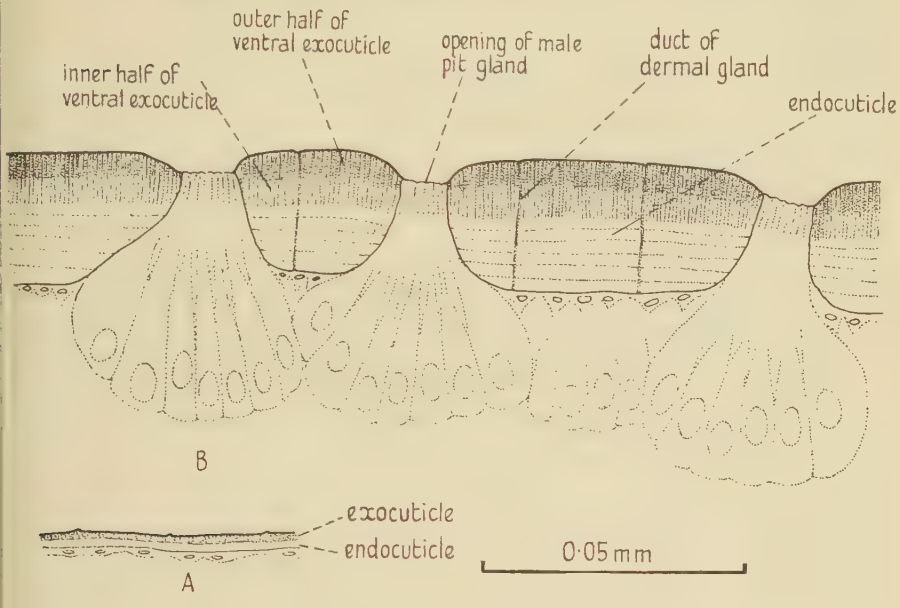
The dorsal cuticle of the abdomen, where it is covered by the elytra, is excessively thin (total thickness 4 μ). The tergites are distinguishable only by their shining surface and faintly amber tint which contrasts with the colourless matt surface of the lateral and intersegmental regions. The varied sculpturing and spicules of the different parts will not be described.

The ventral cuticle is very hard and a deep amber-brown in colour (total thickness in an old insect 36 μ). Evenly dispersed over the sternites are shallow oval depressions each with a tiny slender bristle curving backwards from its anterior end (Text-fig. 1). In the floor of these pits are minute pores—1–3 in the female, a closely packed group of about 25–30 forming a diminutive 'pore plate' in the male. In both dorsal and ventral cuticle the limits of the epidermal cells are clearly indicated in the sculpturing of the cuticular surface. There is a shallow groove between each cell area.

Both tergites and sternites are pierced by the ducts of dermal moulting glands; in the sternites these occur as a circle of some 8–10 glands around

each pit, but they are seen with difficulty in ordinary preparations. They occur also in the pleural regions.

Text-fig. 2 shows histological sections of the dorsal and ventral integument as seen in a recently moulted adult about 6 days old. The cuticle of the tergites (Text-fig. 2A) consists of an excessively thin 'epicuticle', an unstained exocuticle about 1·5 μ thick, and an endocuticle of about 2·5 μ, but no further details can be seen. In the sternites (Text-fig. 2B) the cuticle is made up of a fairly thick amber-coloured 'epicuticle', an amber exocuticle about 12 μ in



Text-fig. 2. Sections through the cuticle of young adult male, about 6 days old. A, dorsal cuticle; B, ventral cuticle.

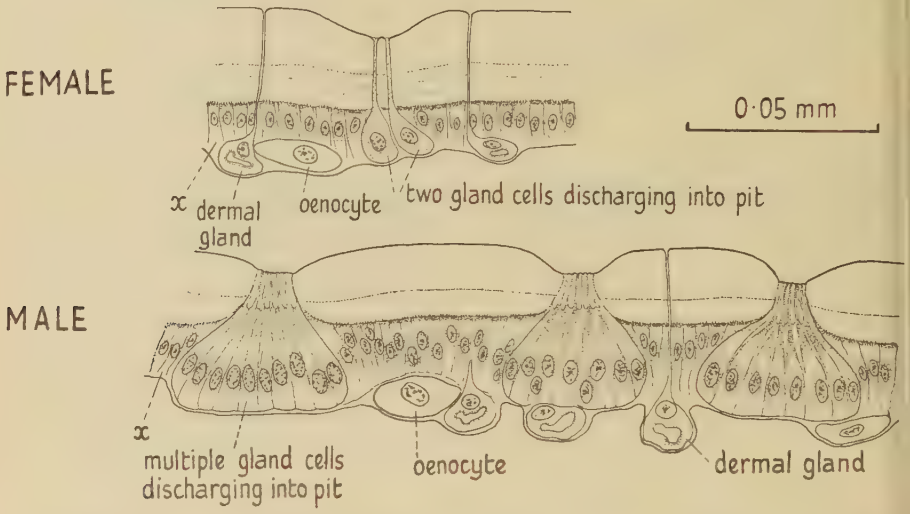
thickness in which the vertical striation of the pore canals is just visible, and a laminated acidophil endocuticle which, in an insect several weeks old, may be double the thickness of the exocuticle. The exocuticle is differentiated faintly into an outer and inner half. The outer half has a slightly greyer shade in the unstained state, and in the recently moulted adult it stains bluish with haematoxylin. At intervals the cuticle is pierced by the ducts of dermal glands, and in the male the groups of pores in the pits form conspicuous interruptions in the sections.

There is the usual complex of cellular elements in the epidermis, best seen in the recently moulted adult (Text-fig. 3). On the sternites, besides the epidermal cells, there are scattered oenocytes, the dermal moulting glands unicellular glands with some three other cells forming the duct, &c. (Hundertmark, 1935)), and the pit glands. Each pore of the pit glands is connected with a single large cell. In the mature male these cells become enormously swollen and the glands project like closely packed buttons far beyond the

surface of the shrunken epidermis.[1] In the female, with usually not more than two pores, the associated gland cells are comparatively inconspicuous.

The structure of the cuticle, particularly the ventral cuticle, has been studied by the methods used on *Rhodnius*.

(i) *Fresh Sections.* In sections cut with the freezing microtome and examined fresh in water the pore canals appear much as in stained sections.



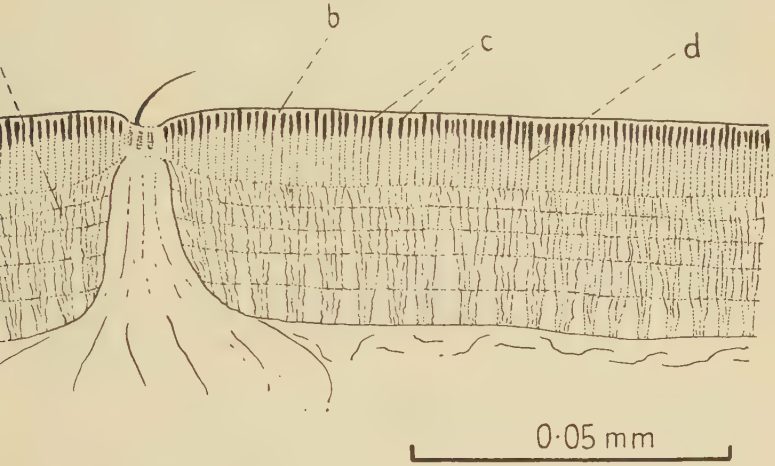
Text-fig. 3. Sections through ventral integument of adult *Tenebrio* 2 days after moulting. *x*, epidermal cells with basiphil apical staining and basiphil filaments extending into the pore canals.

(ii) *Fresh Sections treated with Ammoniacal Silver.* Similar sections, 10 μ thick, were immersed for 1 hour in 5 per cent. ammoniacal silver hydroxide and mounted in Canada balsam. All the pore canals in the exocuticle run a parallel vertical course. In the outer half of the exocuticle their contents stain brown, but only where they come very close to the exposed surface of the sections or where the canals have been actually cut open (Text-fig. 4). These filaments are almost certainly in the form of a close spiral (as described by Richards and Anderson, 1942, in the cockroach), but only here and there can this be resolved with certainty with the light microscope. In the endocuticle the pore canals run a somewhat uneven spiral course, often changing direction as they cross from one lamina to the next and converging gradually towards the base. Throughout the endocuticle they contain a black deposit

[1] These glands resemble those described by Hoffbauer (1892) in the elytra of the Cerambycid *Tetropium*. Hoffbauer noted great differences in closely related species but makes no mention of sexual dimorphism. The glands in *Tenebrio* must presumably have some sexual function. Perhaps in the male they produce an aphrodisiac secretion. It is pointed out to me by Dr. H. E. Hinton of the British Museum, in a personal communication, that hair tufts, presumably the outlets of glands, occur in many male Coleoptera. In other parts of the body (legs, prothorax, &c.) the male *Tenebrio* has only two or three pores in the pits, like the female.

r along their course; and this is not limited to the canals exposed on the of the sections.

staining of the pore canal contents is seen even more clearly if the s after treatment with the silver are bleached for 24 hours in perhydrol; en it is seen that the exposed surface of the exocuticular matrix also weakly with the silver. Beyond the limit of the brown-staining pore in the exocuticle there is an 'epicuticle' apparently devoid of pore but in many places the brown filaments come so close to the surface is not possible to ascribe a measurable thickness to this layer.



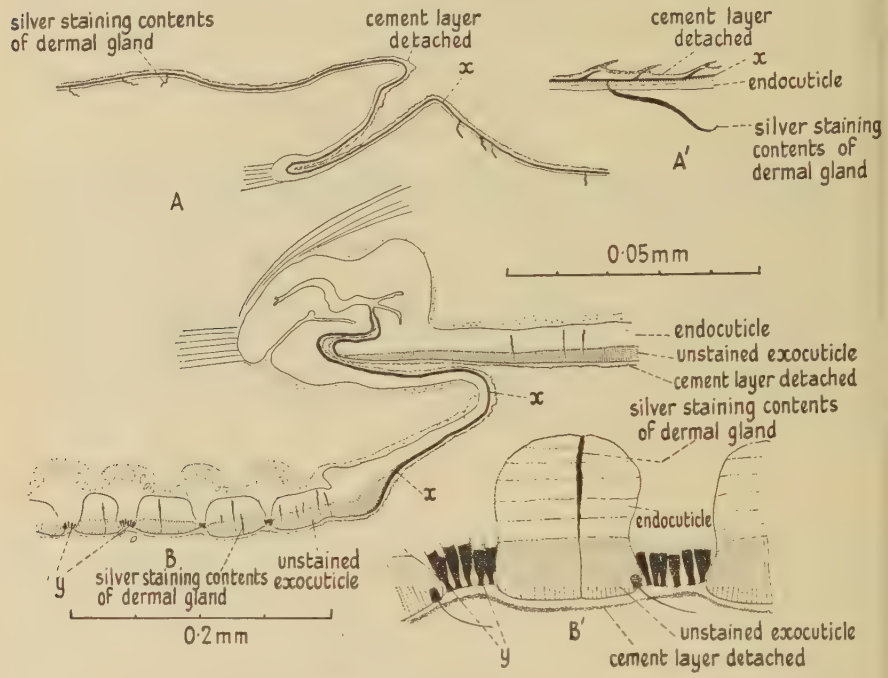
XT-FIG. 4. Section through ventral cuticle of mature adult *Tenebrio*, cut with ng microtome, stained with ammoniacal silver hydroxide and bleached with gen peroxide. *a*, converging convoluted pore-canals in the laminated endocuticle; icuticle', for the most part apparently free from pore canals; *c*, pore canals in the half of the exocuticle with deep brown silver-staining contents; *d*, pore canals in the half of the exocuticle without silver-staining contents.

Fresh Sections dried in Air. Fresh sections were dried thoroughly in air and then mounted direct in Canada balsam. The pore canals of the uticle contain air in many places and appear as black threads. In the icle they contain no air.

Silver Staining after Abrasion with Alumina. If the intact insect is sed in ammoniacal silver hydroxide no staining of the cuticle occurs. rubbed gently on filter paper dusted with alumina and then immersed silver solution, the dorsal cuticle shows a uniform pinkish-brown stain- er all the raised points, with the tips of the pore canals, extending ntly into the substance of the brown-staining membrane, seen in view as minute black points. The ventral cuticle shows no staining lver after abrasion with alumina except at the intersegmental membranes stain in the same manner as the dorsal cuticle.

Silver Staining after Scratching with Glass. If the hard parts of the cuticle are treated more brutally by scratching, with varying degrees

of severity, with a fragment of glass and then immersed in ammoniacal silver, it is again found that where the injury is very superficial the scratches are scarcely visible and there is no silver reduction. Where the scratches go deeper the distal ends of the pore canals stain dark brown in a pale brown background. (There are occasional areas where there is a uniform pale brown staining and the pore canals show up as white points. The explanation of this, which does not appear to be an optical effect, is uncertain.)



Text-fig. 5. Longitudinal sections of cuticle of adult male *Tenebrio*, 1 week after moulting, boiled 5 minutes in chloroform and then treated with ammoniacal silver hydroxide. A, dorsal cuticle and intersegmental joint; A′, detail of same; B, ventral cuticle and intersegmental joint; B′, detail of same. *x*, silver-stained epicuticle and outer exocuticle; *y*, silver-stained cuticle lining the openings of the male pit glands.

(vi) *Silver Staining after Chloroform Extraction.* Extraction with chloroform likewise leads to the exposure of the silver-staining material much more readily in the same regions which are affected by abrasion with alumina. This is most clearly demonstrated by boiling the insect for 5 minutes in chloroform before immersion in the ammoniacal silver. Text-fig. 5 shows longitudinal sections of the dorsal and ventral abdominal cuticle in a *Tenebrio* adult so treated.

The dorsal epicuticle, including the spicules, stains everywhere an intense brown. The same staining extends into the outer part of the exocuticle—sometimes as a uniform brown, sometimes limited to pore canals. The inner half of the exocuticle (stained red with Mann's methyl blue eosin) and the blue-staining endocuticle show no silver reduction.

In the ventral integument, on the other hand, silver staining of the epicuticle is limited to the outer folds of the intersegmental membranes. Elsewhere (apart from the 'cement layer' which will be discussed later) there is no silver staining of epicuticle or exocuticle except in the lining of the orifices of the pit glands or 'pore plates'. The residual contents of the dermal moulting glands also stain deeply and are very conspicuous after this treatment.

(vii) *Chitosan Test.* The ventral cuticle of the fully hardened adult some weeks old was treated with saturated potassium hydroxide at 150° C. until just colourless, then washed in alcohol and examined under a coverslip in surface view. In the deeper layers (endocuticle) the criss-cross fibrils of chitin running in different directions in successive layers are clearly seen; and the areas corresponding with each epidermal cell are evident. In the superficial layers (exocuticle) the pore canals are readily visible; they appear evenly distributed and the cell limits cannot be seen. Now that the amber material has been dissolved away, the pore canals, as seen in optical section, are open to the surface.

If acid iodine is allowed to diffuse slowly in below the coverslip, the matrix of the cuticle in the superficial layers takes on a violet colour, but as this colour spreads it is quite evident that the pore canals remain colourless as minute white points. There is certainly no chitinization of the contents of the pore canals of the exocuticle.

(viii) *Disintegration in Nitric Acid and Potassium Chlorate.* The abdominal cuticle was boiled in chloroform for 10 minutes and then immersed in concentrated nitric acid saturated with potassium chlorate. The dorsal cuticle shows little ebullition as the thin endocuticle dissolves. A very thin epicuticle is left which on warming breaks up with the liberation of small oily droplets.

In the ventral cuticle there is much evolution of gas as the inner layers are dissolved. On warming fairly strongly the exocuticle then fuses and disperses in the form of feebly refractile spheres. If at this stage the preparation is washed with 50 per cent. alcohol and flooded with Sudan B in 70 per cent. alcohol, very few of the droplets stain. They consist presumably of broken-down protein and chitin. There are, however, a few fat-staining droplets, particularly where the epicuticle is beginning to disintegrate. If the heating of the preparations is continued the epicuticle remains last; and as it breaks up, undoubted oil droplets, highly refractile and staining with Sudan B, appear in great quantity. They are far more copious than in the dorsal cuticle, a fact which agrees with the much greater thickness of the epicuticle on the ventral surface as seen in sections.

(ix) *Demonstration of Wax and Cement Layers.* It has not been possible to demonstrate a wax layer in the cuticle of the mature *Tenebrio* adult; the evidence for the presence of this layer will be given when the deposition of the cuticle is described. The existence of a cement layer is readily demonstrated.

If the legs or elytra are immersed fresh in xylene, droplets of water slowly exude from the epicuticle (Wigglesworth, 1942). In many places during this

process a thin layer flakes away in fragments; in other places a delicate continuous membrane, the 'cement layer' is lifted from the surface.

This layer can also be demonstrated by silver staining. If the mature adult beetle is boiled in chloroform for 5 minutes and then treated with ammoniacal silver, a delicate membrane can be seen over the surface of the sternites, extending into the bristle-bearing pits. It consists of very fine evenly dispersed brown granules in a colourless substance. Where the cuticle has been rubbed in mounting it is torn and partially removed; and in sections of such insects it is detached and very easily seen.

Around the openings of the dermal glands, in some preparations, there is a non-staining disk devoid of granules (Pl. I, fig. 1). And extending from the orifice of a certain number of glands (always the glands at the centre of the spaces between the pits, which are a little larger than the others) there is a brown-staining convoluted filament lying on the surface of the cuticle (Pl. I, fig. 2). This appears to be the extruded residue of the secretion from the glands.

A series of adult beetles, 2 weeks after moulting, were subjected to extraction with boiling chloroform for periods of 1, 5, 15, 30, and 60 minutes before treatment with the silver. Insects untreated with chloroform showed no silver staining. All the others showed the granular cement layer over the sternites. The staining was often most intense in the furrows between the cell boundaries on the cuticle surface, giving a net-like appearance (cf. Pl. II, fig. 10). In the insects extracted for longer periods disintegration of the membrane was more advanced; the granules had fused in many places to give larger silver-staining spheres and the convoluted filaments from the dermal glands showed the same change (Pl. I, fig. 3).

It thus appears that the cement layer, as was suggested in *Rhodnius*, also consists of tanned material; but that the silver-reducing groups of the polyphenol concerned are not accessible until the material has been subjected to boiling chloroform. Presumably it is compounded in some way with lipides.

(x) *Summary of the Conclusions on the Structure of the Cuticle.* From these varied observations we may conclude that the dorsal cuticle is not only excessively thin compared with the ventral cuticle, but the substance of the outer layers (the epicuticle and the very thin exocuticle of the delicate sclerites), like the same parts in *Rhodnius*, still contain polyphenol material in a state accessible to the silver solution if the cuticle is cut or the protective coverings on the surface are removed by abrasion or by extraction with lipid solvents.

The thick and brittle cuticle of the sternites has very different properties. It has a relatively thick and hard epicuticle which will not reduce ammoniacal silver (when immersed for 1 hour) even when exposed by abrasion, section, or lipid extraction. The exocuticle is likewise hard and impermeable; polyphenol material easily accessible to the silver solution is confined to the contents of the outer parts of the pore canals, and the limitation of silver staining to the surface of the fresh sections shows how impermeable is the

exocuticle to the diffusion of silver through its substance. (The black deposits in the pore canals of the endocuticle may well be simply a precipitate of the silver by salt or protein.)

The state of the material in the lumen of the pore canals is difficult to determine. In the endocuticle many of the canals contain air on drying; but as Dennell (1947*b*) points out, this does not disprove the existence of solid filaments within them. In the exocuticle they appear to be solid; perhaps their contents consist of protein which has been tanned along with the substance of the exocuticle during the hardening process. It is certain that they do not contain filaments of chitin.

As will be shown later a wax layer covers the cuticulin of the epicuticle, and over the surface of this is a cement layer which likewise consists of tanned materials in intimate association with lipides. In ordinary sections neither cement nor wax layer are optically distinguishable from the cuticulin layer. It has not, therefore, proved possible to homologize the structures here described with the inner and outer epicuticle as defined by Dennell (1946) in *Sarcophaga* larvae and in *Tenebrio* and other adult beetles.

Deposition of the New Cuticle in the Pupa and Young Adult

Outward Changes in the Pupa and Young Adult

Newly moulted pupae of *Tenebrio* were kept at 25° C. Formation of the adult beetle then takes place according to the following time-table. During the first 24 hours the pupa has a glassy appearance and the eyes show only brown central points in the facets; no separation of the adult is visible in the living pupa. By the second day the adult is just beginning to separate from the pupal cuticle, for example, at the tips of the appendages. At 3 days the separation is obvious in the claws, palps, &c., but the new cuticle has not begun to appear. The eyes are just beginning to darken. By 5 days the eyes are fairly dark and the new cuticle is well defined. By 6 days the eyes are quite dark; the claws, tarsal segments, the femero-tibial articulations, and the distal third of the mandibles are darkening. By 7 days the head and legs are becoming dark generally. Moulting to the adult occurs on the eighth day.

The last stages of moulting can be roughly timed by observing the sheaths of the appendages. By the end of the seventh day the limbs are chestnut-brown in colour but the pupal sheaths enclosing them are fully distended with moulting fluid. About 6–10 hours before moulting to the adult, as the inner layers of the pupal cuticle are digested and the moulting fluid absorbed, the sheaths of the last pair of legs begin to collapse, followed by the middle and anterior pairs. Finally, the sheaths around the palps, labrum, and mandibles crumple and collapse, and moulting usually takes place from 2 to 4 hours later. By this time the moulting fluid has disappeared, the pupal cuticle is excessively thin and fragile, and the surface of the insect is quite dry.

The newly emerged adult has the head and prothorax amber; the legs, particularly the joints and claws, are likewise amber, and so are the margins and posterior extremity of the abdomen; but the tergites and sternites of the

abdomen, and the elytra, are alike colourless. Within a few hours the beetle becomes amber all over. After 1 day the general colour is pale chestnut; after 2 days, dark brown; and after 3 days it is fully darkened and almost black.

Histological Changes in the Epidermis and Cuticle

The histological changes have been observed in serial transverse sections of the abdomen fixed with Carnoy's and Bouin's fixatives at all stages, stained with haematoxylin, Mann's methyl blue and eosin, and Mallory's triple stain. Likewise at all stages the dorsal and ventral integument of the abdomen have been removed, freed from underlying tissues, and mounted flat after (i) fixation in Carnoy and staining with haematoxylin; (ii) fixation in Bouin and staining with Sudan black B; (iii) fixation in Altmann and counterstaining with carmine.

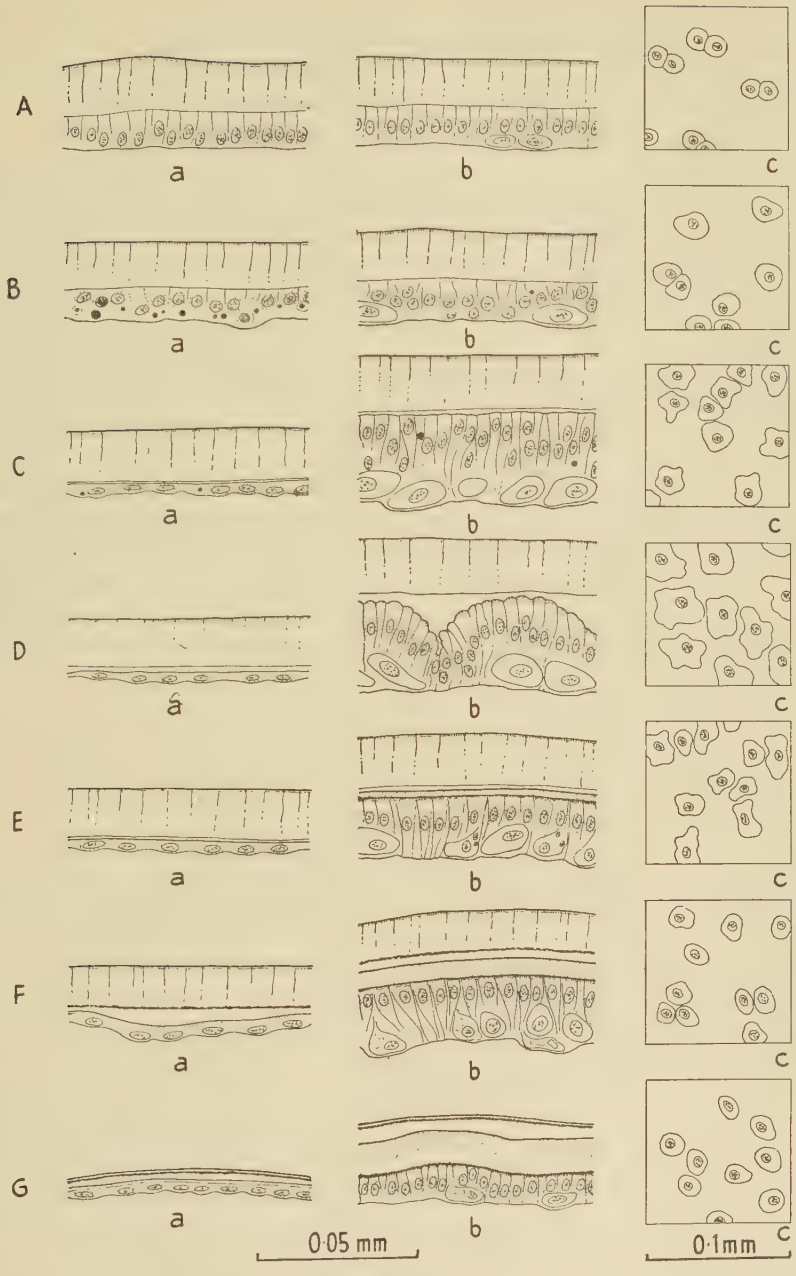
There is no interruption in the process of growth when *Tenebrio* pupates. Within less than 24 hours after moulting mitosis in the epidermis of the sternites has already begun. At this stage there is no obvious difference between the cuticle and epidermis of the dorsal and ventral walls of the abdomen (Text-fig. 6A, *a* and *b*), apart from the fact that oenocytes are confined to the sternites. The epidermis is thin and the nuclei lie all in one plane, not nearly contiguous. The oenocytes of the ventral wall still occur in pairs, embedded among the epidermal cells (Text-fig. 6A, *c*).

By *one day* after pupation a difference is apparent between the epidermis of the dorsal and ventral integument. In the sternites the epidermal cells are now much more numerous, the nuclei are so crowded that they lie in several planes, and there are abundant mitoses. Certain of the nuclei are in chromatolysis (Pl. I, fig. 5). The whole epidermis is in such a violent state of flux that the centres of formation of dermal glands, bristles, &c., cannot be recognized. The oenocytes are still mostly in pairs; they are enlarging slightly and now lie wholly on the inner surface of the epidermis (Pl. I, fig. 6). In the dorsal integument there are no mitoses, no oenocytes, and, as yet, little chromatolysis.

By *two days* (Text-fig. 6B) the same differences between the dorsal and ventral epidermis are becoming exaggerated. That on the dorsal integument is still extremely thin and many of the nuclei are breaking down so that chromatic droplets in all stages of formation are abundant (Pl. I, fig. 4).

By *three days* (Text-fig. 6C) mitosis and chromatolysis in the ventral epidermis is still in active progress; the dermal glands are becoming visibly differentiated and the oenocytes are becoming large and acidophil. In the dorsal epidermis chromatolysis is well advanced and, since no mitosis has occurred, the cells are becoming very sparse as well as very attenuated.

By *four days* (Text-fig. 6D) the epidermis has been detached from the old cuticle, chromatic droplets have almost disappeared, the nuclei are evenly distributed, and the oenocytes are large, lobulated, and strongly acidophil (Pl. I, fig. 7). The innermost layer of the old cuticle stains with haematoxylin



TEXT-FIG. 6. The formation of the adult cuticle in the pupa of *Tenebrio*. *a*, section of dorsal integument; *b*, section of ventral integument; *c*, surface view of oenocytes below the ventral epidermis. A, newly moulted pupa; B, pupa at 2 days; C, 3 days; D, 4 days; E, 5 days; F, 6 days; G, shortly before moulting to adult. The details are given in the text.

and the secretion of the new cuticle is imminent. Particularly in the ventral epidermis the cells now show distinct intercellular membranes and in many places these have a remarkably exact hexagonal arrangement (Pl. I, fig. 8). In preparations fixed with Altmann the great lobulated oenocytes stain dark grey; and they stain conspicuously in Sudan black B.

By *five days* (Text-fig. 6E) (occasionally by 4 days) the new epicuticle is formed. It is refractile and eosinophil, excessively thin on the dorsal surface as compared with the ventral. In ordinary histological sections it appears to be homogeneous although the outer extremity of the cell body lying immediately below is seen to be vertically striated. In the Altmann preparations, in which it can be studied in surface view or in optical section, the epicuticle stains grey and the pore canals are clearly visible as colourless threads in a dark matrix; their outer ends are closed only by an excessively thin refractile membrane. The oenocytes are still large but do not stain so deeply in Altmann.

By *six days* (Text-fig. 6F) the formation of the exocuticle is in progress and the oenocytes are becoming reduced in size; many of them are clearly dying with chromatolysis of their nuclei. The new cuticle, particularly the epicuticle, still stains grey with osmic acid. The arrangement of the epidermal cells, as seen in surface view, is not so regular, but they still lie in rows, this being associated no doubt with the deposition of the parallel strands ('Balken') of chitin extending from cell to cell. Highly vacuolated dermal glands occur in both dorsal and ventral integument.

By *seven days* the exocuticle of the ventral integument is becoming much thicker and its inner and outer halves can be distinguished. For example, with Mallory's stain the inner half stains blue and the outer half stains red, being traversed by blue-staining pore-canal fibrillae which can be traced almost if not quite to the surface of the epicuticle. The dermal moulting glands are even more distended and vacuolated and the pit glands in the male are beginning to enlarge. The oenocytes are reduced in size and stain only a faint grey in osmium tetroxide. The osmic staining of the epicuticle has almost disappeared. Digestion of the inner layers of the pupal cuticle is beginning and is completed by the eighth day (Text-fig. 6G).

In the *young adult* there are no very striking histological changes. The regular arrangement of the epidermal cells is largely lost, but in many places the cells, as seen in surface view, still lie in parallel rows with filaments extending from one to the next as the criss-crossing chitin strands of the endocuticle are formed. As the endocuticle is being secreted the outer parts of the cells stain deeply with haematoxylin, and blue-staining filaments extend into the inner ends of the pore canals. The endocuticle is just about complete in 4 days after moulting. By that time the epidermal cells are small and shrunken and the pit glands of the male are greatly swollen and project far below the epidermis (see Text-fig. 2).

In addition to the sub-epidermal oenocytes described above there are clusters of large oenocytes segmentally arranged in the neighbourhood of the

spiracles (Koch, 1940). These appear to go through the same cycle as the penocytes below the epidermis. They reach their maximum size at about the fourth day of pupal development and appear then to be discharging their secretion into the blood.

Formation of the Epicuticular Layers

(i) *Polyphenol Layer.* The formation of the cuticulin layer of the epicuticle takes place as we have seen between the fourth and fifth days after pupation by the secretion of lipoprotein from the epidermal cells. Upon this is deposited a layer of material rich in silver-reducing substances, the 'polyphenol' layer.

If the epidermis with its new epicuticle, 5-6 days after pupation, is immersed directly in the ammoniacal silver solution there is a clear-cut silver precipitate marking out the membrane between the cell bodies. At the outer limit of each cell there is some finely punctate silver staining, but the epicuticle does not stain. Here and there are single cells in which the outer region stains intensely with the silver (Pl. I, fig. 9) and from the strongly staining cells black filaments run through the pore canals of the newly formed epicuticle to give discrete and evenly distributed black points on the surface.

Soon the black points become general, though always confined to the limits of the cells. It is often possible when examining these preparations in surface view to note the grouping of the pore canals in optical section, to follow them upwards, and to observe that the black droplets on the surface of the cuticle have the same grouping. There can be no doubt that the droplets are formed, as was claimed in *Rhodnius*, by the extrusion of material from the pore canals (Pl. I, figs. 10, 11).

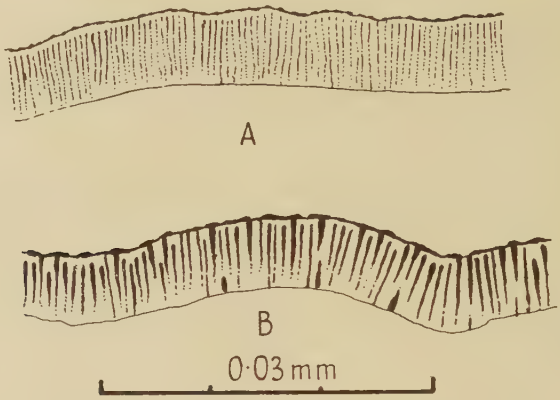
Gradually the exuded droplets enlarge and fuse, so that in pupae in which the new cuticle has been exposed and treated with the silver during the seventh day all intermediate stages can be seen leading up to a condition where the epicuticular cap of each cell is covered by a deep-chestnut-staining layer, more or less continuous, with the punctate black staining of the tips of the pore canals below (Pl. I, fig. 12). Finally this layer joins up with that covering adjacent cells until the cell boundaries almost disappear. At no stage does this superficial polyphenol layer extend over the floor of the pits. Consequently, these show up as oval white spots in the preparations (Pl. I, fig. 12; Pl. II, figs. 4, 5, &c.).

If, during this process, the exposed cuticle, before treatment with the silver, is wiped with a piece of filter-paper cut to a point, the silver-staining material will run together into rounded droplets. If it is more strongly rubbed with filter-paper, the material may be removed completely, leaving only the black-staining tips of the pore canals.

These observations, made upon whole preparations seen in surface view, can be confirmed in sections. Text-fig. 7 shows sections of the new cuticle which had been exposed in the 6-day pupa and treated with silver. In Text-fig. 7A the cuticulin layer is visible as a clear zone beyond the silver-staining

filaments in the exocuticle. But here and there these filaments cross the clear zone, becoming continuous apparently with the polyphenol droplets on the surface. At the stage represented in Text-fig. 7B the polyphenol is being actively secreted; it appears chiefly in the form of tapering filaments passing outwards through the cuticle, with their pointed end leading, to fuse upon the surface. The form of these filaments bears a striking resemblance to that assumed by droplets of biliverdin as they pass through the striated border of the Malpighian tubes in *Rhodnius* (Wigglesworth, 1943*b*).

(ii) *Wax Layer.* During the last few hours before moulting the polyphenol layer is largely covered over. Soon after the silver staining has reached its



TEXT-FIG. 7. Section of new cuticle in 6-day-old pupa. Whole insect immersed for 1 hour in ammoniacal silver hydroxide before fixation. Description in text.

greatest intensity and become practically continuous, it begins to break up into rounded patches with non-staining areas between; and gradually the staining spots become reduced in number and in size (Pl. II, fig. 1). Around each spot can be seen a pale-brown halo. This is due to the diffuse staining of the exocuticle and the dark-brown staining of the filaments in the pore canals, the distal limits of which now lie on a distinctly lower plane than the dark-brown spots (Pl. II, fig. 2)—though sections through the dark spots often show brown filaments running from them into the pore canals.

These observations show that at this stage silver can get into the deeper layers of the cuticle only through the silver-staining patches; the intervening areas are impermeable. Having got in it will diffuse laterally through the exocuticle to reach adjacent pore canals. The slightest abrasion of the surface with alumina, or brief immersion in chloroform, will expose the polyphenol layer everywhere.

It would appear that, as in *Rhodnius*, the polyphenol layer is being covered by a layer of lipoid or wax immediately before moulting. At the time of moulting droplets of water will not leave a waxed pipette to adhere anywhere to the surface of the adult cuticle; but after extraction for 5 minutes in boiling chloroform wetting is notably increased. At this stage there is still plenty of

silver staining in the form of scattered rounded spots, particularly along the side of the abdomen and on the intersegmental membranes. Here and there the stained spots lie in rows, mostly running in an antero-posterior direction (Pl. II, fig. 3). These have evidently resulted from abrasion during moulting and show how fragile is the protective layer at this time. Within 2 hours the silver staining is much reduced, though what remains has the same distribution as before. By 6 hours after moulting there is a further marked diminution, but even after 24 hours there are occasional scattered spots on the intersegmental and marginal regions.

(iii) *Cement Layer.* Meanwhile the cement layer is being poured out over the surface of the wax. At the time of moulting, droplets of water, at the tip of a very fine waxed pipette, will not adhere to the surface of the sternites. After 1 hour they usually begin to adhere slightly, particularly in the lateral regions. When brought into contact with the cuticle the droplet on the pipette breaks and a very tiny drop remains on the insect. After 6 hours the droplets will adhere all over the cuticle with an angle of contact ranging from 90° upwards, but they never spread over the surface. This slightly increased affinity for water takes place in spite of the fact that the hardening process that is going on in the substance of the cuticle will be tending to make it hydrophobe; it is certainly the result of the outpouring of the cement layer.

The cement comes from the dermal glands. We have seen that after extraction with boiling chloroform it will stain with ammoniacal silver, and this property may be utilized to demonstrate its secretion. Adult *Tenebrio* in the act of moulting and at periods of 30 minutes, 1 hour, 2 hours, &c., afterwards have been fixed briefly in Carnoy, boiled in chloroform for 5 minutes, and then immersed in the ammoniacal silver.

At the time of moulting the sternites stain deeply after this treatment; excepting the floor of the pits, the dark-brown colour is continuous and extends for the most part right across the abdomen. The contents of the dermal glands stain black (Pl. II, figs. 4, 5, and 6). The gland vesicle has a convoluted form tapering to a point, and in many of the glands the secretion appears to be pressed into a distended mass near the orifice. Towards the sides of the abdomen in some places the dense polyphenol staining of the exocuticle is becoming discontinuous (Pl. II, fig. 7); and where that happens the dermal glands are partially emptied and there is some superficial dark-brown silver staining, concentrated particularly in the furrows between the cell areas. Occasionally, around the mouths of the ducts of individual glands, there are rounded areas where the staining of the exocuticle is becoming covered; clearly the glands are just beginning to extrude their secretion.

At 30 minutes after moulting there is hardly any increased wetting of the cuticle by water. Staining with ammoniacal silver now shows, particularly in the lateral regions, that the cement is being discharged, with the result that the polyphenol layer is no longer exposed by chloroform extraction. Where this process is most advanced the glands are empty; where the polyphenol staining is still continuous, as in the middle of the segments, the glands are

distended as at moulting; and all intermediate stages occur (Pl. II, figs. 8 and 9). The new cement layer shows regular fine brown granules in a colourless matrix.

One hour after moulting only the middle regions of the sternites show intense silver staining. The lateral parts stain a diffuse brown. In the central area the dermal glands still show intense black contents. In the lateral parts they are completely empty, the polyphenol layer is entirely covered, and the only silver staining is in the granular cement. This extends over the whole surface, including the floor of the pits and the bristles, but it is most intense in the furrows between the cellular impressions. Here it may form more or less continuous brown lines, giving a net-like appearance (Pl. II, fig. 10). In the intermediate zone occasional dermal glands can be seen in which the black-staining contents are in process of discharge.

After 6–8 hours the dermal glands are mostly emptied and the cement layer is evident everywhere. There is little change at 1 day after moulting. At 2 days after moulting the black-staining filaments discharged from the largest of the dermal glands have made their appearance (Pl. I, fig. 2) and practically all the glands are emptied.

It was interesting to apply this technique of staining with silver after extraction with boiling chloroform to *Rhodnius* at the time of moulting. In the *Rhodnius* fifth-stage nymph there are two sorts of dermal glands (Wigglesworth, 1933): type 'B' present in great numbers, particularly around the bristle-bearing plaques, with a large distended oval vesicle; and type 'A' much less plentiful, with an elongated intracellular vesicle. In the nymph at the time of moulting it is only type 'A' whose contents stain black with silver after chloroform extraction; the vesicle contents of type 'B' are unstained. If the preparation is allowed to dry on the slide after dehydrating in alcohol the vesicles of type 'B' fill with air and on mounting in Canada balsam they show up very conspicuously.

In order to see whether glands of this type had been overlooked in *Tenebrio* a preparation of a newly moulted *Tenebrio* adult was likewise allowed to dry in the air, but no glands were revealed. It appears that in *Tenebrio* there is one type only, and its contents reduce silver after chloroform extraction.

It appears from these observations that the cement layer in *Rhodnius* resembles the substance of the oötheca in the cockroach, as described by Pryor (1940*a*), in being the product of two glands, one of which secretes a protein solution and the other a polyphenol. When the cement in *Rhodnius* is first discharged it is strongly hydrophil and droplets of water spread actively on the surface (Wigglesworth, 1947*a*). In *Tenebrio* there is only a slight increase in this adhesion of water when the cement is secreted. This is probably because the cement in *Tenebrio* contains more lipoid material; for it is the product of a single type of gland, the contents of which reduce silver only after extraction with boiling chloroform.[1]

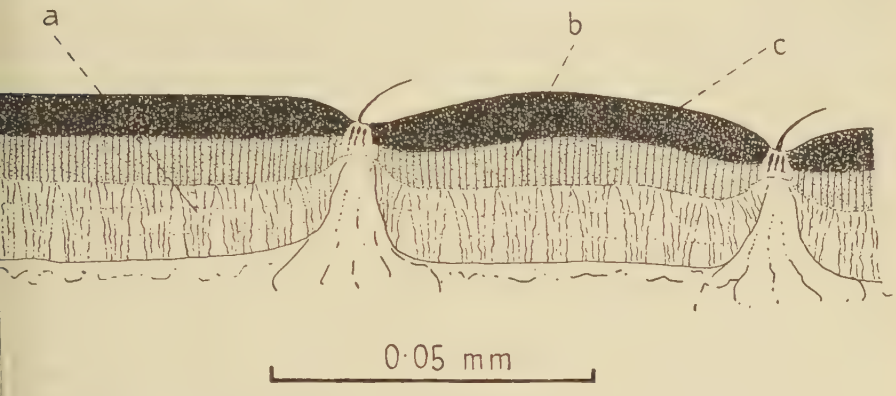
[1] It is unlikely that the glands opening into the floor of the pits in *Tenebrio* contribute to the cement. There is a very great difference in their form and development in the two sexes and they do not appear to reach full activity until some days after moulting.

Hardening of the Cuticle

No attempt has been made to follow the movements and distribution of the enzymes concerned in the hardening and darkening of the cuticle (cf. Dennell, 1947*a*).

The most striking feature of the hard ventral abdominal cuticle of *Tenebrio* as compared with the cuticle of *Rhodnius* is the failure of the epicuticle and of the substance of the exocuticle to reduce silver once hardening is complete.[1]

Text-fig. 8 shows a fresh section of the cuticle in the young adult one day after moulting, when hardening is still far from complete, cut with the freezing



Text-fig. 8. Section of ventral abdominal cuticle of adult *Tenebrio* 1 day after moulting, cut with the freezing microtome and immersed fresh in ammoniacal silver hydroxide. *a*, laminated endocuticle partly formed; pore canals slightly converging; *b*, inner half of exocuticle showing rather feeble silver staining; *c*, outer half of exocuticle showing intense silver staining.

microtome and treated with ammoniacal silver. The outer half of the exocuticle is stained a uniform intense blackish-brown in which the pore canals can scarcely be differentiated. The inner half also stains brown, but not so deeply, and the pore canals are more distinct. The endocuticle is about equal in thickness to the exocuticle. The lamination is faintly visible and the slightly converging pore canals are brown-stained throughout their course. The much stronger silver reduction in the outer half of the exocuticle agrees with the limitation of polyphenol to the outer half of the pore canals in the fully hardened cuticle (p. 200). Perhaps this outer half represents the amount of exocuticle that has been laid down at the stage when the most active secretion of polyphenol is taking place.

The process of hardening in the sternites is most readily studied by rubbing the cuticle surface gently with alumina before immersion for 1 hour in the silver (Wigglesworth, 1945). If this is done at the moment of moulting the wax layer is removed and everywhere there is a strong diffuse staining of

[1] This difference is one of degree. Immersion in ammoniacal silver for 1 hour has been used in the test, and this gives only a very faint staining of sections of the exocuticle. On prolonged immersion more intense reduction would doubtless be obtained.

the exocuticular matrix and an intense black staining of the pore canals (Pl. II, fig. 11). At 6 hours after moulting, when the cuticle is just beginning to darken, the abraded areas stain dark brown as before, but in the form of small spots instead of extensive rounded patches (Pl. II, fig. 12). The exocuticle still stains readily, but now the cement has been laid down the covering is less fragile. At 1 day after moulting the silver staining after abrasion consists of faint pink areas with the punctate brown staining of the tips of the pore canals distributed through them, while in the fully hardened beetle at 4 days as we have seen, there is no silver staining of the sternites after abrasion with alumina.

These results indicate that although silver-reducing material is probably still present in the epicuticle and exocuticle, the substance of these layers in the fully hardened insect has become so impermeable that the silver does not have access to it.

Along with the hardening, the exposed parts are blackened. If fragments of the fresh cuticle of the newly moulted insect are immersed in dilute ferric chloride, the exocuticle develops a diffuse violet coloration. This is most evident in the parts which will become darkened. Perhaps it indicates the concentration of tyrosine in the cuticle of these regions. The cuticle removed at an earlier stage (in pupae at 6 or 7 days) and immersed in dilute ferric chloride gives a diffuse greenish coloration which changes to red in the presence of alkalies. This colour is said to be given by ortho-dihydroxy phenols only when they are in solution (Lison, 1936). No definite coloration has been obtained with the 'polyphenol layer' on the surface of the cuticulin. Perhaps the phenol responsible for the silver reduction is bound to protein and is insoluble.

Waterproofing of the Cuticle at the Time of Moulting

Pupae of *Tenebrio* were kept at 25° C. in dry air and weighed daily. Among 12 pupae with an average weight of 97·7 mg. (85–119) the loss of weight per diem was about 1·3 mg. The loss in the young beetles after moulting ranged from 1·2 to 1·8 mg. per diem with a mean of about 1·6 mg. During the 24 hours which included the moult the loss of weight (excluding the dry weight of the cast skin) varied widely from 4·1 to 12·6 mg., with an average of 7·7 mg.

In three of these insects weighings were made during the act of moulting in order to estimate the relative importance of the water lost with the skin and that lost by transpiration afterwards. The results were as follows:

	A	B	C
Total loss of water during 24 hours from moulting, in mg.	11·8	6·4	8·0
Water lost with the skin	4·2	3·0	2·5
,, ,, by transpiration	7·5	3·4	5·5
Weight of the dry cast skin	1·2	1·5	1·1

Weighings of insects at intervals after moulting showed that the increased rate of transpiration occurs chiefly in the early hours after shedding the skin but here again there is great individual variation. For example, the insect

above, which lost 3·4 mg. by transpiration in 24 hours, lost 1·2 mg. in the first 6 hours, 2·2 mg. in the next 18 hours; while the insect C, which lost 5·5 mg. in 24 hours, lost 3·2 mg. in the first 3 hours and 2·3 mg. in the next 21 hours.

These results agree with the observations on the newly moulted *Tenebrio* which show a variable amount of silver staining that disappears during the 24 hours after moulting. They are entirely consonant with the view that waterproofing results from the deposition of a layer of wax over the polyphenol.

In *Rhodnius*, during the day on which moulting occurs, the water loss is rather more than doubled (Wigglesworth and Gillett, 1936). Smallman (1942), in the case of *Dixippus*, found that the rate of loss was increased about four times but returned to normal within one day. The values in *Tenebrio* likewise show an increased loss on the day of moulting about four to six times that of the beetle during the succeeding days.

I am indebted to Mrs. A. Whittingham for a large amount of careful technical assistance and to Mr. F. J. Bloy for taking the photomicrographs.

Summary

The conclusions on the structure of the cuticle in *Tenebrio* have been summarized on p. 204.

Observations on the deposition of the cuticle are in general agreement with those made on *Rhodnius*.

Mitosis and chromatolysis precede the formation of the definitive epidermis. The basic layer of the epicuticle, 'cuticulin', is then laid down. It consists of condensed lipoproteins (subsequently tanned, it is supposed, by quinones) and its deposition is immediately preceded by the peak in the secretory cycle of the subepidermal oenocytes.

Pore canals from the epidermal cells penetrate the cuticulin layer and pour out silver-reducing material (believed to be dihydroxyphenols in insoluble form) upon its surface. This material is confined to the areas overlying the cell bodies during all but the last stages in its formation, when it fuses to give a more or less continuous layer.

During the last few hours before moulting a wax layer appears to be laid down over this polyphenol layer. By the time moulting occurs the polyphenol layer is almost covered and the insect is nearly waterproof. During the first day after moulting, while the secretion of the wax is being completed, the loss of water by transpiration is about four to six times the normal.

Very soon after moulting the dermal glands discharge the cement layer over the surface of the wax. The substance of this layer and the contents of the dermal glands reduce ammoniacal silver after extraction with boiling chloroform. It is suggested that it consists of polyphenol-containing material associated with protein and lipides.

(It is shown that in *Rhodnius* the cement layer is formed by the admixture of secretion from the two types of dermal gland previously described. The

one produces a solution of protein, the secretion of the other agrees in properties with that here described in *Tenebrio*. The similarity of this arrangement to that discovered by Pryor in the colleterial glands of the cockroach is pointed out.)

In addition to these cement glands there are glands of unknown function opening into the floor of the pits in the cuticle. These are highly developed in the sternites of the male, small and inconspicuous in the female.

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EXPLANATION OF PLATES

Plate I

All figures at the same magnification with 4-mm. objective except figs. 9, 10, and 11 taken with 2-mm. objective.

Fig. 1. Surface view of ventral abdominal cuticle. Boiling chloroform 5 minutes, followed by ammoniacal silver hydroxide. The black spots are the ducts of the male pit glands (cf. Text-fig. 5*y*). The cement layer is visible as a granular film; it shows colourless round areas and at the centre of each of these is a black point which is the opening of a dermal gland.

Fig. 2. The same. Towards the left the silver-stained cement layer has been partially rubbed off, leaving unstained areas. Silver-staining convoluted filaments protrude from the mouths of some of the dermal glands; others appear as small black spots.

Fig. 3. The same, but treated in boiling chloroform for 30 minutes. The cement layer is fusing into rounded silver-staining droplets. Above and below are convoluted filaments which have partially fused.

Fig. 4. Epidermis of dorsal abdominal integument in the 2-day-old pupa seen in surface view. Ehrlich's haematoxylin. Many of the nuclei are breaking down with the formation of chromatic droplets.

Fig. 5. Epidermis of ventral abdominal integument in the 1-day-old pupa seen in surface view. Ehrlich's haematoxylin. Numerous mitoses; abundant and crowded epidermal cells; some nuclei breaking down to form chromatic droplets.

Fig. 6. The same. More superficial view (from inside) showing the oenocytes, many of them still in pairs. Numerous chromatic droplets.

Fig. 7. The same in 4-day-old pupa showing the oenocytes very large and lobulated

Fig. 8. The same, showing the very regular arrangement of the nuclei of the epidermis. These cells are partially obscured by the large oenocytes which overlie them.

Fig. 9. The epidermis and new epicuticle of the abdominal sternites in the 5-day-old pupa treated with ammoniacal silver hydroxide. In the lower part the finely granular silver staining of the cells can be seen. The polygonal black areas are individual cells staining intensely with the silver. Black filaments run upwards from them into the new cuticle.

Fig. 10. Surface view of the new ventral cuticle in 6-day-old pupa treated with ammoniacal silver hydroxide. Silver-staining material occurs in the form of minute dots on the surface. In many places these are enlarging and fusing to form irregular blotches. The cell areas are clearly marked out.

Fig. 11. The same field as fig. 10 at a slightly lower focus showing in optical transverse section the silver-staining filaments in the pore canals. The cell boundaries can be seen in places but are not so distinct.

Fig. 12. Surface view of the new ventral cuticle in 7-day-old pupa treated with ammoniacal silver hydroxide. Polyphenol layer almost complete over the cell areas; mostly absent along the intervening regions. This silver-staining layer is absent also from the pits which appear as oval white spots.

Plate II

Magnifications as in Plate I, all taken with 4-mm. objective, except fig. 2, taken with 2-mm. objective, and fig. 4 taken with 16-mm. objective.

Fig. 1. Surface view of the new ventral cuticle in late 7-day-old pupa treated with ammoniacal silver hydroxide. Polygonal areas corresponding with individual cells are visible here and there, but for the most part the polyphenol layer is covered and only occasional rounded spots stain. (The punctate areas are the openings of the male pit glands.)

Fig. 2. Detail of the same showing the punctate staining of the pore canals around the silver-staining spots.

Fig. 3. Adult in act of emerging from the pupa treated with ammoniacal silver hydroxide. Only a few scattered spots stain. To the right is presumably a mechanical abrasion of the covering wax layer.

Fig. 4. Newly moulted adult, treated 5 minutes in boiling chloroform before immersion in ammoniacal silver hydroxide. Low power view of ventral abdominal integument from inside. The polyphenol layer gives a continuous dark stain except in the pits, which show as white spots with black centres (the male pit glands). The black convoluted contents of the dermal glands are conspicuous.

Fig. 5. Newly moulted adult female treated as fig. 4. Surface view of ventral abdominal integument. The white spots are the pits. The black spots are the contents of the distended dermal glands.

Fig. 6. The same. Dorsal abdominal integument. The black objects are the silver-staining contents of the dermal glands. The two white spots are companiform sensilla.

Fig. 7. Recently moulted adult treated as fig. 4. Exposure of the polyphenol layer is incomplete even after chloroform extraction.

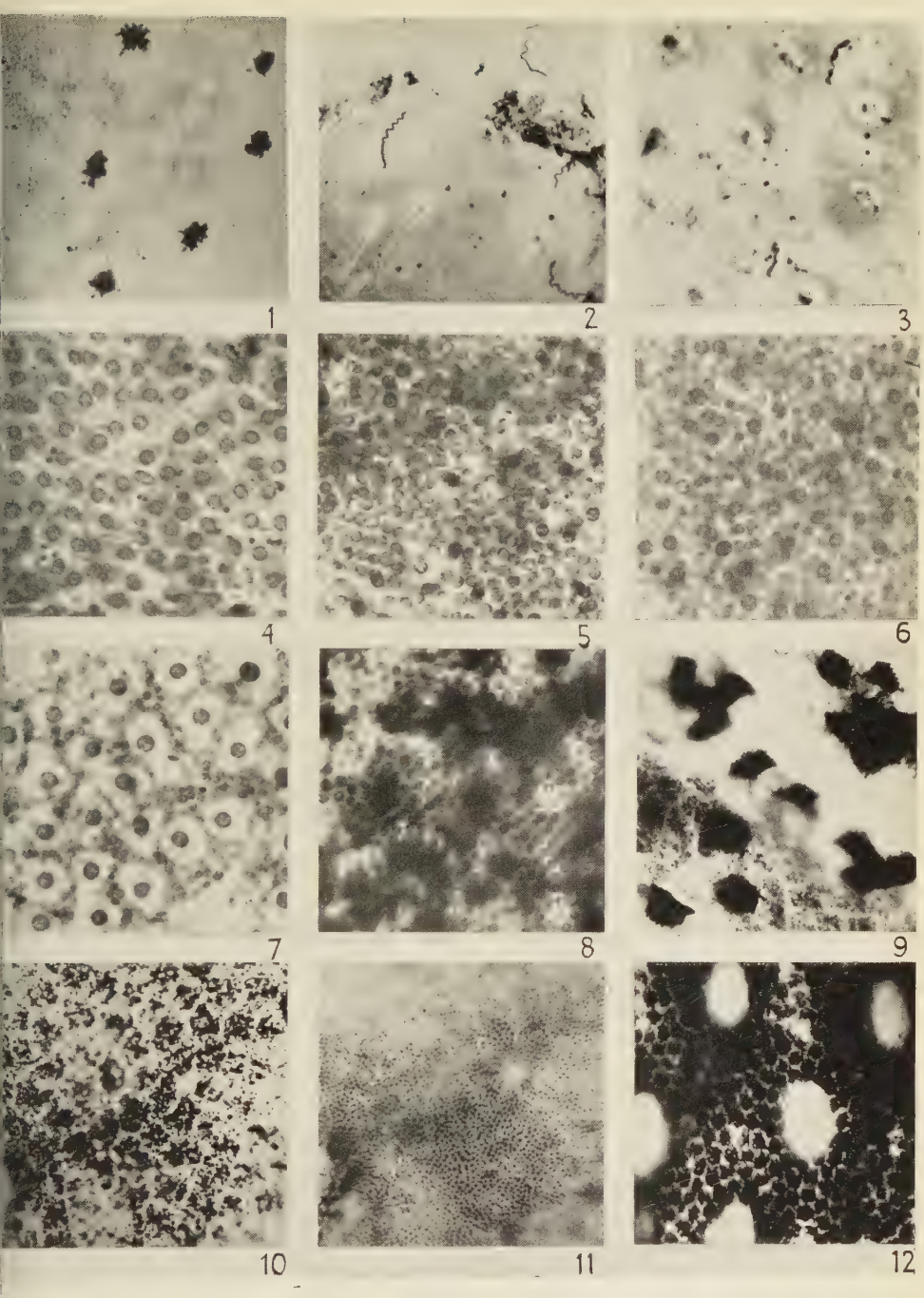
Fig. 8. Adult male 30 minutes after moulting, treated as fig. 4. Above and to the right the cement layer has been discharged; there is a finely granular superficial staining, the dermal glands are empty, and the polyphenol layer stains only in a few rounded spots. Below and to the left the staining of the polyphenol layer is almost continuous; the dermal glands, still filled with secretion, can be faintly seen through the cuticle.

Fig. 9. A preparation at the same stage similar to Fig. 8. Above and to the left the finely granular cement layer can be seen, with darker lines along the intercellular boundaries; the polyphenol layer is exposed only in rounded spots. Below and to the right there is continuous staining of the polyphenol layer (except in the pits) and the distended dermal glands are faintly visible below.

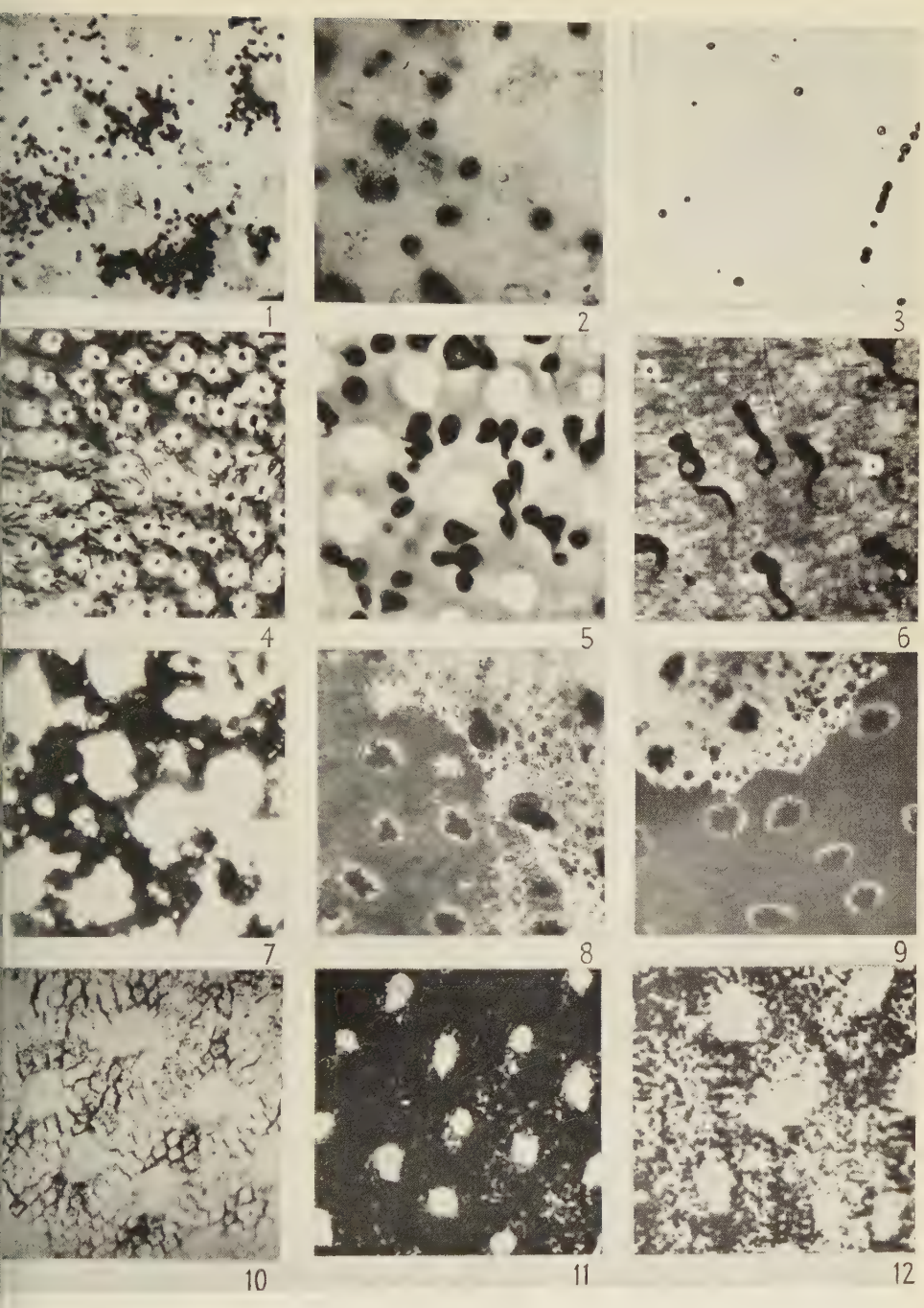
Fig. 10. Adult female 1 hour after moulting, treated as fig. 4. Dermal glands emptied. No staining of polyphenol layer. Superficial silver staining of the granular cement layer, particularly along the intercellular boundaries.

Fig. 11. Newly moulted adult; ventral cuticle gently rubbed with alumina and immersed directly in ammoniacal silver hydroxide. Almost continuous staining of polyphenol layer except in the pits and here and there along the intercellular boundaries.

Fig. 12. Adult 6 hours after moulting; same treatment as fig. 11. Silver staining in the form of small spots.



V. G. WIGGLESWORTH.—PLATE I



V. G. WIGGLESWORTH.—PLATE II

A Study of the Cytoplasmic Components during the Gametogenesis of *Bos taurus*

BY

R. A. R. GRESSON, PH.D., D.SC.,

AND

I. ZLOTNIK, PH.D., M.R.C.V.S.

(*From the Department of Zoology, University of Edinburgh*)

With one Plate and fourteen Text-figures

INTRODUCTION

THIS paper is an account of the Golgi material and mitochondria of the germ-cells, and of spermateleosis, of domestic cattle (*Bos taurus*). Zlotnik (1943) described a nuclear ring present in the spermatids and spermatozoa of the bull, but so far as the writers are aware there is no previous account of the cytoplasmic components during the gametogenesis of this animal. Much of the literature on the cytoplasmic components of the germ-cells of mammals has been discussed recently by Gresson and Zlotnik (1945) and by Zlotnik (1948).

MATERIAL AND METHODS

The ovaries of three heifers, aged from 9 to 11 months, were fixed in Champy-Kull, Kolatchev, and Aoyama. For the examination of the Golgi material pieces of ovaries fixed according to the method of Kolatchev were the most satisfactory. Small portions of the ovaries of two cows, aged 4 years, were fixed in Susa and Bouin.

For the study of the male germ-cells pieces of the testes of three bulls of varying ages were fixed in Flemming (without acetic), Kolatchev, and Aoyama. The sections fixed in Kolatchev were stained with acid fuchsin or with haematoxylin.

In all cases the animals were killed and small pieces of tissue placed in the fixing fluid as speedily as possible. Sections were subsequently cut at 5 μ and at 8 μ in thickness.

OBSERVATIONS

Oogenesis

All stages of the development of the primary oocyte were represented in the material examined, and in addition oogonia were visible in some of the sections. The history of the oocyte is of the usual mammalian type. The older primary oocytes possess a well-marked zona radiata. Follicles in process of rupture were not observed.

The Golgi Material

Oogonia. The Golgi material is a compact mass which occupies a juxta-nuclear position. Owing to its small size and compact nature it was not possible to determine its structure with certainty, but it is probable that it is composed of Golgi elements arranged in a manner similar to those of the young primary oocyte.

Oocytes. The Golgi material of oocytes surrounded by a few elongate follicle-cells is situated at one side of the nucleus (Pl. 1, fig. 1). Careful study of this material indicates that, in the oocyte of the cow at least, it is not in the form of a network, but consists of spherical and rod-shaped bodies. In oocytes surrounded by a single layer of columnar follicle-cells the position of the Golgi material varies. In some oocytes it is still localized at one side of the nucleus; in some of these cells deeply impregnated granules and rods are present in the cytoplasm, particularly in the neighbourhood of the nucleus. The Golgi material of a few cells consists of two parts, usually situated at opposite sides of the nucleus, and of rods and granules scattered through the cytoplasm (Pl. 1, fig. 2). The sequence of events is as follows. When the follicle-cells become columnar, the juxta-nuclear Golgi material spreads out over part of the nucleus. Very soon granules and small rod-shaped Golgi elements leave the localized mass and are distributed through the cell. At a slightly later stage large masses of Golgi substance, as well as small bodies, move out through the cytoplasm.

Two large masses of Golgi material are still present in some of the cells surrounded by two layers of follicle-cells. They rapidly break up into smaller bodies, and shortly after a third layer of follicle-cells is formed the Golgi substance consists of small granules in the central cytoplasm, and of large bodies situated in the peripheral region of the cell (Pl. 1, fig. 3).

With the further growth of the follicle, small spaces appear within the follicular epithelium and the zona radiata is formed. Oocytes at this stage are of two types as regards the arrangement of the Golgi material and the number of fat globules present. In one type, globules of fat are relatively few, and the Golgi material is composed of comparatively large rods and granules lying at the periphery of the cell, and of very fine granules scattered throughout the cytoplasm. In the second kind of oocyte the Golgi bodies in the peripheral region are very much smaller (Pl. 1, fig. 4). In these cells fat globules are much more numerous than in oocytes of the first type, and the majority are located in loose clumps which may extend from the periphery to the central region. The number of fat globules present and the form of the peripheral Golgi bodies closely resembles the condition of the oocytes of Graafian follicles (Pl. 1, fig. 5). It is concluded, therefore, that these oocytes are at a later stage of development than those possessing comparatively few fat globules and larger Golgi bodies.

Follicle-cells. The Golgi material of the young elongate follicle-cells is a small compact mass at one side of the nucleus. When the cells become columnar, the Golgi substance is sometimes situated at the pole of the nucleus

next to the oocyte, but in many cells it is still located at one side of the nucleus (Pl. 1, fig. 2). At a slightly later stage, the Golgi material of all the cells lies between the nucleus and the oocyte. In older follicles, which are still composed of a single layer of cells, the Golgi substance is less compact and is visibly composed of rods and granules which surround a clear area.

In follicles consisting of two layers, the Golgi material is similar in appearance to that of the preceding stage, but its position in relation to the nucleus varies. The Golgi material of the majority of cells in the layer adjacent to the oocyte is at the pole of the nucleus next to the oocyte, in a few cells it is at the side of the nucleus, and in others it is situated in the end of the cell directed away from the oocyte. In all the cells of the outer layer the Golgi material is located at the pole of the nucleus directed towards the oocyte. In follicles composed of three layers, the Golgi substance of most of the cells of the layer nearest to the oocyte is localized at the pole opposite to the oocyte, but in a few cells it is still situated at the side of the nucleus. In the other two layers, the Golgi material is directed towards the oocyte.

In follicles composed of several layers, and in Graafian follicles, the Golgi material of the majority of the cells of the two layers nearest to the oocyte are directed away from the oocyte; in the cells of the other layers it is directed towards the oocyte.

The Golgi material of the cells of older follicles is frequently less compact than in very young follicles and is often visibly composed of granules and rods. In many of the larger follicles, bodies, which are probably granules of secretion, are present in contact with the Golgi substance and in the surrounding cytoplasm.

Mitochondria

Oogonia. The mitochondria are very fine granules which surround one pole of the nucleus. They are very faintly stained and, owing to their small size, are often difficult to detect.

Oocytes. The mitochondria of oocytes surrounded by a single layer of elongate follicle-cells are, like those of the oogonia, minute granules which are only clearly visible in certain parts of the sections. They surround one pole of the nucleus and extend to the equatorial region. At a slightly later stage, when the follicle-cells are broader but are still arranged with their long axes parallel to the oocyte, the mitochondria begin to spread out through the cytoplasm. When most of the follicle-cells have become columnar, the mitochondria are scattered in groups throughout the cell; the majority have increased in size and many now stain deeply.

In oocytes surrounded by two layers of follicle-cells, the mitochondria are more numerous and the majority are larger than during the preceding stage. They are absent from the vicinity of the nucleus, but are distributed fairly evenly throughout the rest of the cell.

The mitochondria of oocytes situated in follicles consisting of several layers are arranged in a broad peripheral zone. They vary considerably in size;

many are large granules or small spheres, while others are in the form of fine granules. When the small cavities within the follicular epithelium run together to form the antrum, the mitochondria begin to move away from the periphery, so that groups extend from the peripheral layer towards the central cytoplasm (Pl. 1, fig. 6).

In oocytes of Graafian follicles, the mitochondria form a large mass which lies in the central cytoplasm and is in contact with one pole of the nucleus. A few small groups of granules are scattered through the cell and in most cases surround fat globules (Pl. 1, fig. 7).

Follicle-cells. The mitochondria were badly preserved and were often invisible; in a number of cells, however, fine granular mitochondria were recognized. In the young elongate follicle-cells, the mitochondria are at one side of the nucleus. At a later stage they are situated between the nucleus and the oocyte. When the follicle consists of several layers, the mitochondria of the cells of the first layer lie in the part of the cell which is directed away from the oocyte, while those of the cells of the other layers are in the cytoplasm nearest to the oocyte. In the older follicles, the cytoplasm of many cells, except those in contact with the oocyte, is drawn out into a long process which extends towards the oocyte and contains mitochondria and frequently secretory granules.

Yolk

Small globules are visible in young oocytes surrounded by a single layer of columnar follicle-cells. Since these are deeply blackened in Kolatchev preparations and are represented by clear spaces in material fixed in Susa fixative, they are identified as fat globules. In the older oocytes, the globules increase in size and number, and in the oocytes of Graafian follicles several large globules, usually surrounded by small Golgi elements and by mitochondria, are present, chiefly towards the periphery. The reduction in the size of the Golgi bodies, at the time when there is a marked increase in the amount of fat, suggests that the peripheral Golgi material may play some part in the formation of the globules.

The young oocytes at first contain a single nucleolus and a number of small bodies which stain in a similar manner. Later, more than one nucleolus is present and the small bodies are more numerous. As the small bodies are often in contact with nucleoli, it is possible that they originate as buds from the latter. The presence of bodies of similar size and staining properties in the vicinity of the nuclear membrane and within the cytoplasm of oocytes surrounded by one or more layers of follicle-cells suggests that the buds are extruded from the nucleus. If these bodies are nucleolar extrusions, it is probable that they contribute to the nutritive material of the egg. As they stain in a similar manner to the mitochondria their history could not be followed in the material available, even in oocytes fixed in Bouin's fluid in which the mitochondria are imperfectly preserved. Cytoplasmic bodies were not present in late oocytes fixed in Susa fixative; this may indicate that the

nucleolar extrusions are used up in the formation of nutritive material. Unfortunately, young oocytes were not present in the ovaries fixed in Susa.

Spermatogenesis

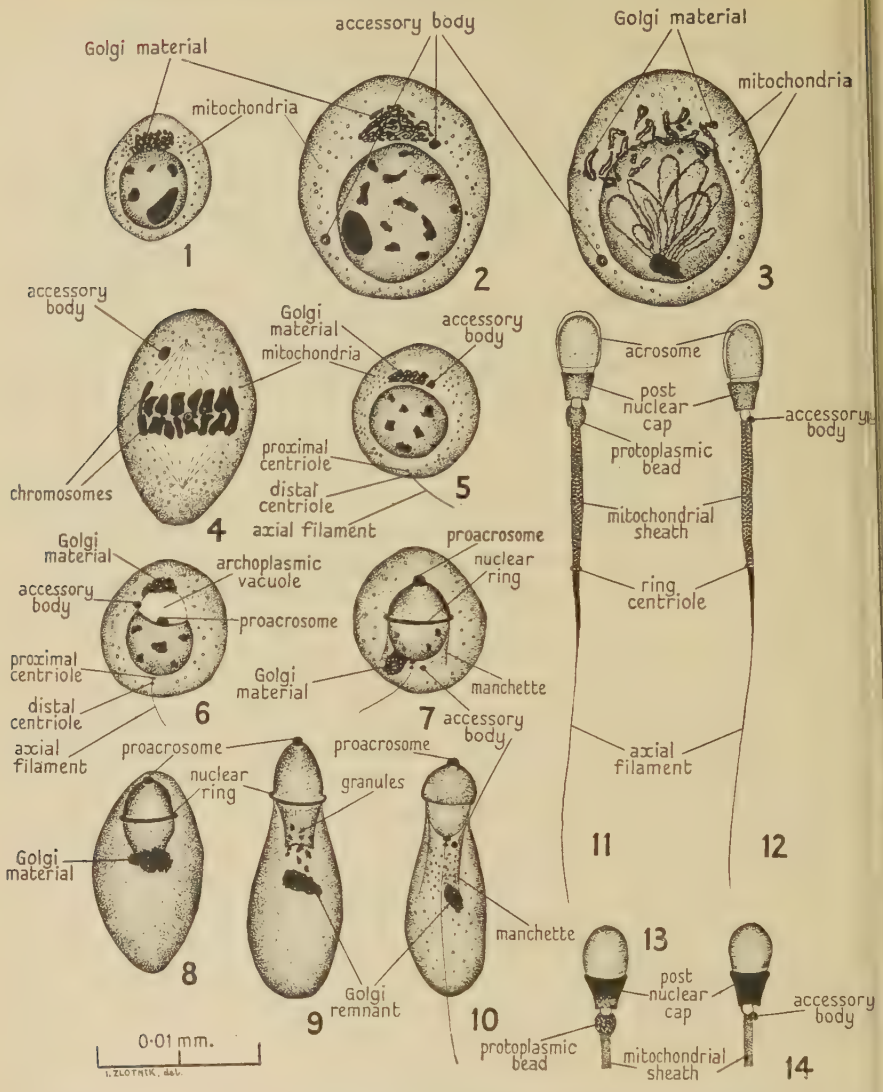
The history of the cytoplasmic components during the spermatogenesis of the bull in general resembles the history of these structures in other mammals investigated by the writers (Gresson, 1942; Gresson and Zlotnik, 1945). It is unnecessary, therefore, to give a lengthy account of the stages of spermatogenesis.

Spermatogonia. The Golgi material is made up of rods and granules which lie at one pole of the nucleus (Text-fig. 1). Each element consists of a deeply impregnated cortex and a lightly impregnated central region. The mitochondria surround the nucleus and are most numerous in the immediate vicinity of the Golgi material.

Spermatocytes. In the resting primary spermatocytes the Golgi material is localized, and granular mitochondria are scattered round the nucleus. A body which blackens with osmium tetroxide is visible close to the Golgi material of many of the primary spermatocytes (Kolatchev preparations); bodies of approximately the same size, but somewhat lighter in colour, are often present elsewhere in the cytoplasm (Text-fig. 2). Similar bodies are present in Flemming preparations; they are identified as accessory bodies.

During the prophase of the first spermatocyte division, the Golgi material becomes less compact. Many of the Golgi elements are larger than those of the spermatogonia and younger spermatocytes, and the cortical and central regions are more clearly visible (Text-fig. 3). The mitochondria become scattered through the cytoplasm, and remain in this condition throughout the subsequent stages of division (Text-fig. 4). During the metaphase and anaphase the Golgi material consists of two widely separated groups of elements lying towards opposite poles of the cell. Spermatocytes at late prophase were not identified in Kolatchev and Aoyama preparations, but observations indicate that the separation of the Golgi elements into two groups takes place during the late prophase or early metaphase. Accessory bodies are present in the cytoplasm during the stages of division (Text-figs. 3 and 4).

Spermateleosis. The Golgi material of the young spermatid is localized at one pole of the nucleus; the mitochondria are scattered through the cytoplasm but are slightly more numerous in the neighbourhood of the Golgi material than elsewhere in the cell. An accessory body is often visible close to the Golgi material (Text-fig. 5). At a later stage an archoplasmic vacuole, containing an archoplasmic granule, is present. The acrosome arises from these structures (Text-figs. 6–10). When the Golgi material migrates to the posterior pole of the nucleus the elements of which it is composed become closely clumped together (Text-figs. 7 and 8). It remains for some time in contact with the posterior pole of the nucleus. Later, argentophilic and osmiophilic granules appear in the vicinity of the posterior part of the nucleus



Text-figures 1, 2, 3, 5, 6, 7, and 10 from Kolatchev preparations. Text-figures 4, 11, and 12 from Flemming preparations. Text-figures 8, 9, 13, and 14 from Aoyama preparations.

TEXT-FIG. 1. Spermatogonium.
TEXT-FIG. 2. Primary spermatocyte.
TEXT-FIG. 3. Primary spermatocyte—early prophase.
TEXT-FIG. 4. Primary spermatocyte—early anaphase.
TEXT-FIG. 5. Young spermatid.
TEXT-FIGS. 6–8. Spermatids.
TEXT-FIGS. 9 and 10. Late spermatids.
TEXT-FIG. 11. Spermatozoon.
TEXT-FIG. 12. Spermatozoon showing accessory body; protoplasmic bead not shown.
TEXT-FIG. 13. Spermatozoon.
TEXT-FIG. 14. Spermatozoon showing accessory body; protoplasmic bead not shown.

(Text-fig. 9). As some of these granules are often in contact with the Golgi material it is probable that they originate from it. Two centrioles were identified. The proximal one is large and soon comes into contact with the posterior pole of the nucleus; the distal centriole passes to the end of the middle-piece where it persists as the ring centriole (Text-figs. 11 and 12). The nuclear-ring arises on the rim of the nuclear depression produced by the archoplasmic vacuole (Text-figs. 6–9). The manchette marks out the cytoplasm which is included in the middle-piece of the spermatozoon (Text-fig. 10).

The mitochondrial sheath of the middle-piece of the spermatozoon is formed by the majority of the mitochondria of the late spermatid which become concentrated inside the manchette (Text-figs. 10–12). The post-nuclear cap is deeply impregnated in silver preparations. A protoplasmic bead, with argentophilic granules on its surface, is situated at the proximal end of the middle-piece (Text-figs. 11 and 13). An accessory body is present in the neck region of the spermatozoon (Text-figs. 12 and 14).

Discussion

The present work indicates that the localized Golgi material of the oogonia, oocytes, spermatocytes, and spermatids of domestic cattle is not a network; this agrees with comparatively recent observations on the germ-cells of other mammals (Gresson, 1940 and 1942; Gresson and Zlotnik, 1945; Zlotnik, 1948). The method of dispersal of the Golgi material of the young oocyte varies in different animals. The young oocyte of the cow appears to differ from those of the other mammals studied in that, prior to its dispersal, the Golgi material separates into two masses which come to lie at opposite sides of the nucleus.

Henneguy (1926) states that in follicles of the guinea-pig consisting of a single layer, the Golgi material is oriented towards the oocyte. In follicles of several layers, the position of the Golgi material varies, and in the discus proligerus it lies between the nucleus and the follicular cavity. He believes that the cells at first secrete a substance which is passed into the oocyte, and later take part in the formation of the follicular liquid. The location of the Golgi material in the follicle-cells of the mouse led Gresson (1933) to support Henneguy's suggestion. More recently it has been shown that the Golgi substance of the follicle-cells of other mammals undergoes a change of polarity correlated with the growth of the follicle. Beams and King (1938) state that, in the guinea-pig, the Golgi material 'is generally polarized towards the egg or towards the follicular cavity—a condition which is taken to indicate that the more physiologically active pole of the cell is marked by the position of the Golgi apparatus'. The work of Aykroyd (1938) on the human oocyte, and of Zlotnik (1948) on the oocytes of the dog, the cat, and the rabbit, demonstrates that the Golgi material of many of the cells in the older follicles undergoes a change of polarity and is often situated in relation to the accumulation of the follicular liquid.

If the location of the Golgi material is an indication of functional polarity then its change of position in the follicle-cells of the cow indicates that these cells at first form a secretion which is passed into the oocyte, and later produce a substance which accumulates in the follicular cavity. The presence of secretory granules in the vicinity of the Golgi material of cells in late follicles supports this view, but the migration of granules into the follicular cavity was not observed. In certain mammals the passage of secretory granules from the follicle-cells into the oocyte has been recorded (Zlotnik, 1948). The mitochondria undergo changes of position which are probably correlated with the functional polarity of the cell.

The behaviour of the mitochondria of the oocyte of the cow is in general similar to that of the mitochondria of other mammals. In oocytes of Graafian follicles, however, the majority form a large mass in the central cytoplasm while in the other mammals studied the mitochondria are fairly evenly scattered throughout the cytoplasm, or else there is a peripheral concentration in addition to small groups situated in the central region of the cell.

As regards yolk-formation, there is no direct evidence that the cytoplasmic components of the oocyte of the cow participate in the process. Yolk globules are frequently surrounded by Golgi bodies and by mitochondria, and it may be that these structures are concerned with the elaboration of the globules. Extrusion of nucleolar material to the cytoplasm takes place, and possibly adds to the nutritive material of the ovum. The literature on yolk-formation in the mammalian egg is conflicting; Zlotnik (1948) has recently discussed this process in some mammals.

The more recent work on the cytoplasmic components of the male germ-cells of mammals is reviewed by the writers in a previous paper (Gresson and Zlotnik, 1945). In the present contribution, therefore, certain aspects of spermatogenesis only are discussed.

The time at which the Golgi material of the spermatocyte is dispersed varies in different mammals. In the bull the Golgi elements do not scatter through the cytoplasm, as in some animals, but separate into two groups during the late prophase or early metaphase.

Wadsedalek (1914) records the presence of a 'chromatoid body' in the germ-cells of the bull. The writers believe that this structure is an accessory body. Accessory bodies are present in the spermatocytes and spermatids of other mammals, and are believed by Gresson and Zlotnik (1945) to originate from the localized Golgi material and to give rise to the Golgi substance of the spermatozoon.

The argentophilic and osmiophilic granules present at the posterior end of the nucleus of the late spermatid most probably originate from the Golgi material, and some of them, at least, form the granules of the protoplasmic bead. It is possible that some of the granules may take part in the formation of the post-nuclear cap.

Spermatozoa within the seminiferous tubules of the bull possess a protoplasmic bead. This agrees with previous observations of Gresson and Zlotnik

(1945). Gatenby and Collery (1943) state that two beads are present on the spermatozoon of the dog and the guinea-pig, and that the lower one (protoplasmic bead) is not present on sperms within the testis but only on sperms from the epididymis. The writers found that the protoplasmic bead of the other mammals examined by them is eliminated after the spermatozoa enter the epididymis. Smears from the epididymis of the bull were not examined.

The present work confirms previous observations of Gresson and Zlotnik (1945) that the distal centriole does not divide, and that the manchette enters into the formation of the middle-piece of the spermatozoon.

We wish to express our thanks to Professor James Ritchie for research facilities, and to Mr. J. R. Fant for taking the photomicrographs.

Summary

Oogenesis

1. The Golgi material of the oogonium and the early primary oocyte consists of spherical and rod-shaped bodies situated at one side of the nucleus. In older oocytes it is present as rods and granules distributed through the cell.

2. Granular mitochondria surround the nucleus of the oogonium and the early primary oocyte. In older oocytes they are scattered in groups through the cytoplasm. Later, they are arranged in a broad peripheral zone. In oocytes of Graafian follicles the majority of the mitochondria are situated in the central cytoplasm.

3. Small fat globules are present in young oocytes. In older oocytes the globules increase in size and number. There is a reduction in the size of the Golgi elements at the time when there is a marked increase in the amount of fat; this suggests that the Golgi material plays a part in the formation of the globules. Nucleolar material appears to be passed into the cytoplasm; it may contribute to the formation of nutritive material.

4. The position of the Golgi material of the follicle-cells suggests that the cells at first form a secretion which is passed into the oocyte, and later a secretion which enters the follicular cavity. Secretory granules are visible in many of the cells of late follicles. The position of the mitochondria appears to be correlated with the functional polarity of the cell.

Spermatogenesis

1. The Golgi material of the spermatogonium, resting spermatocyte, and early spermatid consists of rods and granules situated at one side of the nucleus. The Golgi elements separate into two groups during nuclear division. Argentophilic and osmiophilic granules present at the posterior pole of the nucleus of the late spermatid probably originate from the Golgi material. The Golgi remnant is eliminated with the residual cytoplasm.

2. Accessory bodies are present in primary spermatocytes and in spermatids. An accessory body is present in the neck region of the spermatozoon.

3. The mitochondria are granular; their behaviour during spermatogenesis is described.

4. The formation of the acrosome and the nuclear-ring is briefly described.

5. The distal centriole passes undivided to the posterior end of the middle-piece. The manchette marks out the cytoplasm which is included in the middle-piece.

6. The protoplasmic bead, present at the anterior end of the middle-piece, is formed while the spermatozoon is within the testis.

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DESCRIPTION OF PLATE I

Photomicrographs. Figs. 1–5 from Kolatchev preparations. Figs. 6 and 7 from Champy–Kull preparations.

Fig. 1. Young oocyte showing localized Golgi material. × 960.

Fig. 2. Young oocyte; the Golgi material has separated into two masses situated at opposite sides of the nucleus. The Golgi material of some of the follicle-cells is shown. × 960.

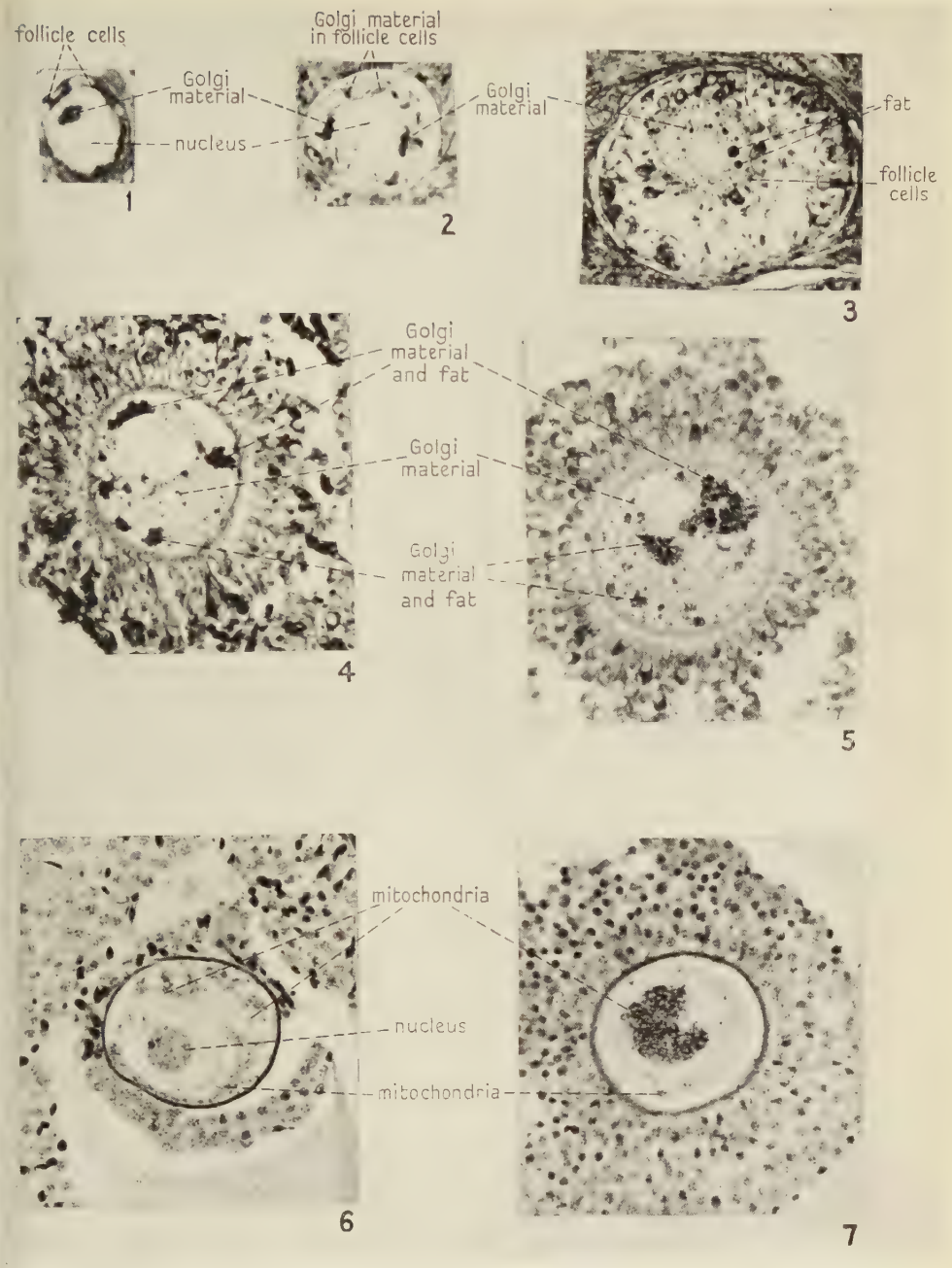
Fig. 3. Oocyte showing scattered Golgi bodies and a few fat globules. × 480.

Fig. 4. Late oocyte showing fat globules surrounded by Golgi bodies. Golgi bodies are also scattered through the cytoplasm. × 480.

Fig. 5. Oocyte of Graafian follicle showing fat globules and Golgi bodies. × 480.

Fig. 6. Late oocyte to show mitochondria. × 480.

Fig. 7. Oocyte in Graafian follicle to show mitochondria. × 480.



R. A. R. GRESSON AND I. ZLOTNIK—PLATE I

An Easily Controlled Method for Staining Mitochondria

BY

A. J. CAIN

(*From the Department of Zoology and Comparative Anatomy, Oxford*)

THE great disadvantages of the standard staining methods for mitochondria are that they are very complicated and cannot be applied to different tissues until suitable times for the action of the various stains have been worked out by a long process of trial and error.

The following method is easy to control and apply. Destaining and restaining are quickly carried out, times may be worked out on a single section, and, as each reagent is used for a single purpose (not, for example, as a simultaneous differentiator and stain), each stage may be controlled separately. Mitochondria are red, cytoplasm is pale blue or colourless, nuclei are medium blue, and plasmosomes (nucleoli) pink or red.

1. Fix small pieces in Helly's fluid for 6 hours.
2. Postchrome for 48 hours at 37° C. in a saturated aqueous solution of potassium dichromate.
3. Wash overnight in running water.
4. Embed in paraffin wax and cut sections of a suitable thickness (about 3 μ). Bring the sections down to water, treating with iodine ($\frac{1}{2}$ in 70 per cent. alcohol) and then sodium thiosulphate (5 per cent. aqueous solution) on the way.
5. Dry a slide except where the sections are, flood with acid fuchsine in aniline-water, and heat gently until 'steaming', in order to overstain, exactly as for Altmann's technique. (For detailed instructions see Baker, 1945, p. 190.)
6. Wash off the acid fuchsine with distilled water, observe the section under the high power of the microscope, and irrigate with an alkaline solution. One drop of saturated aqueous sodium carbonate solution in 10 c.c. of distilled water gives a fairly rapid differentiation, taking 30 sec. to 1$\frac{1}{2}$ minutes.
7. To stop differentiation and brighten the acid fuchsine, dip the slide into 1 per cent. hydrochloric acid. For the criteria of differentiation see below. If brightening is undesirable because extraction of the dye from the cytoplasm is difficult, wash in distilled water instead of dipping into the acid.
8. When the slide has been correctly differentiated, wash it in distilled water, then counterstain progressively in a $\frac{1}{2}$ per cent. aqueous solution of water-soluble methyl blue. The stain 'Soluble Blue' (not 'soluble blue, crystals AS') sold by B.D.H. is suitable. For a discussion of methyl blues see below. Wash off the stain with distilled water, dip in 1 per cent.

acid for 3 seconds only, wash with distilled water, go through the alcohols (in no great hurry), and mount in Canada balsam.

After the acid fuchsine has been applied, the section is brilliant red throughout, though it may be possible to see that the mitochondria are darker than the rest of the cytoplasm. Differentiation should proceed until the mitochondria are dark red against pink cytoplasm. Some cells (e.g. nephridial cells of the leech *Glossiphonia complanata* (L.)) will give up all the acid fuchsine from the cytoplasm before the mitochondria begin to pale. Others (e.g. intestinal epithelium of the mouse) retain a certain quantity, but it is easily masked by the methyl blue. For such cells as the former, comparatively thick sections (5–10 μ) may be used, but for the latter they should be thin (3 or 2 μ). If differentiation is carried too far, the section can be restained in acid fuchsine.

When the staining and differentiation of the acid fuchsine are satisfactory, the nucleus and cytoplasm are counterstained with a methyl blue dye. This dye, being acid, does not interfere with the differentiation of the acid fuchsine. Many basic dyes or dye-lakes can be used to differentiate acid dyes. For example, Kull (1914) used toluidine blue to differentiate acid fuchsine, but as the blue had overstained the section before completing differentiation, he used aurantia to differentiate the differentiator, thereby producing his well-known trichrome method.

The trouble with such procedures is that the times of suitable differentiation and suitable staining by a combined stain and differentiator are unlikely to be the same, and it is difficult to control a differentiation proceeding in a coloured solution. Trichrome methods give beautiful results when properly carried out, but dichrome methods are perfectly satisfactory for almost all purposes, and with a colourless differentiator are far easier to control. Quite good preparations can be made from mouse small-intestine epithelium (and probably from other tissues) by overstaining in acid fuchsine, and staining and differentiating simultaneously with Ehrlich's haematoxylin, tap-water (which is not sufficiently alkaline to disturb the acid fuchsine) being used for blueing. A suitable time in the haematoxylin for 3 μ sections of this tissue is 7 minutes. But the cytoplasm is not very clear and the contrast with the red in the mitochondria is rather poor. Methyl green (a basic dye) may be used as in Bensley's method (1911) but it is exceedingly rapidly removed by alcohol and very sensitive to acid balsam. Methyl blue (water soluble) when acting as an acid dye is superior because differentiation of the acid fuchsine can be carried out separately and it will not interfere, it can be made to stain nuclei well, it gives good colour-contrast with the mitochondria, and it is not sensitive to alcohols and acid balsam. (It is removed by strong alcohol, but, as with acid fuchsine, only after periods of some hours.) If overstaining occurs it can be removed, but much more rapidly, by the same differentiator as was used for acid fuchsine, followed by 70 per cent. alcohol.

The group of dyes usually called methyl blue (excluding methyl blue SS) are closely related acid triphenylmethane dyes. They can behave as acid or as

basic dyes according to the circumstances of their preparation and the pH of the media in which they are dissolved. In alkaline solutions they are dull blue, rather slowly staining, acid dyes, rather easily removed by alcohol. In acid solution they are intensely blue basic dyes fast to alcohol. When dissolved in distilled water they may behave as acid or as alkaline dyes according to the circumstances of preparation. Various samples were examined, and it was found that those that are sold in the form of crystals with a reddish-bronze lustre and give intensely reddish-blue solutions in distilled water behaved as basic dyes, and those sold as dark-blue powders giving a less intense and rather dull-blue solution in distilled water behaved as acid dyes. B.D.H. Soluble Blue is an example of this class. An acid dye is essential to avoid further differentiation of the acid fuchsine, which is rapidly removed by the basic methyl blue dyes. Use of a basic methyl blue in an alkaline solution cannot be recommended because, if the solution is sufficiently alkaline for the dye to be acidic, then it will itself differentiate the acid fuchsine. It is therefore essential to use an acid dye in distilled water. As the colour produced by it is purplish and rather feeble, the dye can be made much more intense and less purple in tone by dipping the slide into 1 per cent. hydrochloric acid for a few seconds only. If the slide is left in it for longer, the methyl blue, now basic, will attack the acid fuchsine. Intensification is complete in 2 or 3 seconds, and the slide is immediately removed and freed from acid by washing with distilled water.

Methyl blues are fairly readily removed by 70 per cent. alcohol when in the alkalinized state, that is, when behaving as acid dyes. If a valuable slide has been overstained with methyl blue, it can be destained by a minute's treatment with sodium carbonate solution followed by a quarter of an hour in 70 per cent. alcohol, and may then be restained with acid fuchsine and methyl blue.

The method described has given satisfactory results with the intestine, liver, and kidney of the mouse, and nephridial cells, muscle-fibres, and gut epithelium of the leech *Glossiphonia*. Kidney should be cut if possible at 2 μ, as the mitochondria are clustered so thickly together. Suitable times for differentiation and counterstaining are best worked out on the first slide of each batch, and then applied to the rest. The time for methyl blue varies greatly with the thickness of the section, thicker sections requiring much less staining than thinner ones. 3 μ sections of mouse intestine required 1 minute, 10 μ sections only 30 seconds.

Summary

A method for staining mitochondria is described in which sections are overstained with acid fuchsine, differentiated in sodium carbonate solution, and counterstained with a methyl blue acting as an acid dye.

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A New Method for Oblique Microscopical Illumination

BY

JOHN R. BAKER

(From the Department of Zoology and Comparative Anatomy, Oxford)

With two Text-figures

THE purpose of the technique described in this paper is to provide the easiest possible means of controlling the direction and degree of obliquity of the light used to illuminate microscopical objects.

The method is applicable when striations or other regularly-repeated markings on objects are situated so close together that, with axial illumination, the diffraction-spectrum they produce lies partly or wholly outside the back lens of the objective. A clear image can in these circumstances only be produced by throwing the direct light to one side of the back lens, and thus making room for the spectrum on the other side. Methods for achieving this object are familiar to microscopists. Advantages are claimed for the new method described in this paper. The intention is to allow the microscope to be used at its ultimate resolving-power with the greatest possible convenience and effectiveness. The method is likely to be useful in the testing of high-power objectives and in the study of the minutest markings on the shells of certain diatoms.

The basis of the method is that the *objective* of the microscope is used as an *eyepiece* through which the direction and degree of obliquity of the light are examined while the mirror is tilted in various ways. The condenser has to be in a special position in order to enable the objective to be used in this way.

No special apparatus is required. The source of light must be rather bright: an ordinary 100-watt filament-lamp with 'pearl' glass is very convenient. ('Opal' glass does not let through enough light.) Two stops with circular apertures are needed, with some easy means of changing from one to the other. One stop should have an aperture about $3\frac{1}{2}$ or 4 mm. in diameter, the other about 15 mm. These will be called respectively the small and large stops. The stop in use must be placed immediately in front of the lamp. (An iris diaphragm can be used instead, but is rather less convenient.) The lamp must be arranged in such a way that a line drawn from the centre of the mirror through the centre of the stop would pass through the brightest part of the lamp. It is also desirable that this line should be approximately at right angles to the optical axis of the microscope. A stainless steel mirror is preferable to a glass one, because it gives only a single image, but it is by no means a necessity for the successful working of the method. An oil-immersion condenser of wide aperture (such as Watson's Holoscopic) is required; it must be accurately

centred and the iris diaphragm below it must be kept wide open. An oil-immersion objective of high aperture is of course necessary. The eyepiece used should be fairly powerful (I use a Watson's Holoscopic × 14). It is useful to have also a short cork that fits the top of the draw-tube, with a hole about 8 mm. in diameter bored through the middle of it. The use of this will be mentioned at the appropriate place.

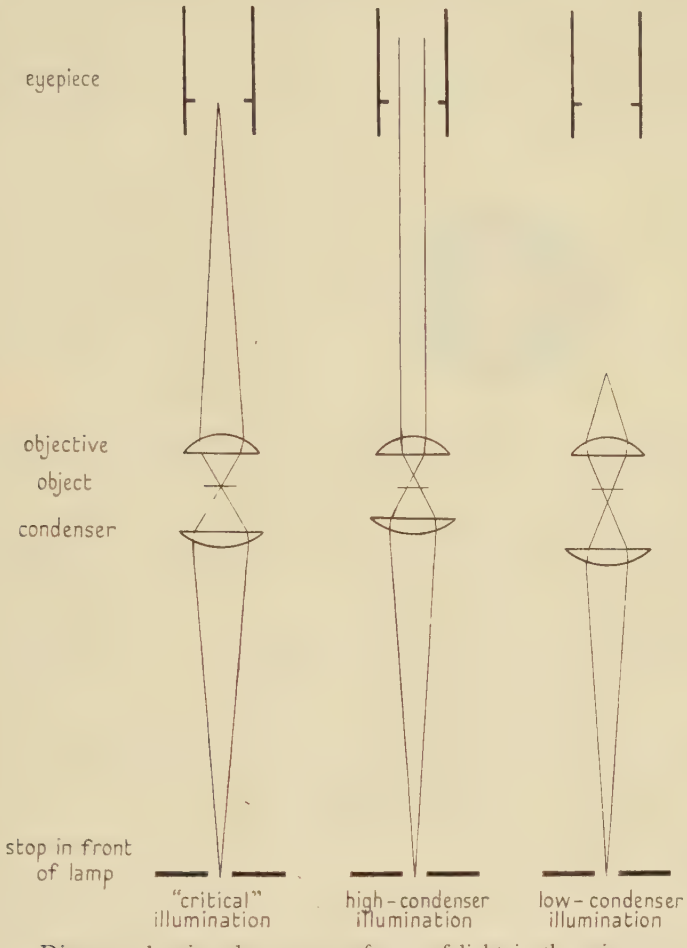
The method for showing the striations of the shell of *Amphipleura pellucida* will now be described as an example. As is well known, this elongated diatom is transversely striated, the lines being exceedingly regular and closer together than the shortest wave-length of visible light. It is best to have the diatoms mounted in hyrax. Choose a specimen that lies somewhat apart from others so as to avoid complications arising from the appearance of more than one spectrum. (The presence of part of another diatom near the edge of the field of view is not harmful.) It does not matter in what direction the chosen diatom is orientated. In this description I shall suppose, for simplicity, that it happens to lie with its long axis in the 'north and south' direction. The striations, which will at first be invisible, will be directed east–west.

Place the small stop in front of the lamp and by means of the condenser focus the image of the stop so that it is clearly seen while the outline of the diatom is exactly in focus. (It is best to use a low-power eyepiece at this stage.) The illumination is now 'critical' (see Text-fig. 1). Next remove the eyepiece and substitute the bored cork. Put the large stop in front of the lamp, and with one hand hold a sharp pencil with its point in the centre of the stop. Hold the eye near the cork (the purpose of which is to make it easy to keep the line of vision axial) and look down the tube of the microscope. Accommodate the eye for distant vision. (Most microscopists do this reflexly when they look through a microscope. If special glasses are used for distant vision, they should be worn.) *Now focus the condenser slowly upwards until the point of the pencil is clearly seen.* Stop focusing upwards directly this is achieved. If you go too far, focus down again. The proper position has been achieved when a very small movement of the condenser downwards would cause the pencil-point to appear violently distorted and then vanish.

You have now secured what I call 'high-condenser illumination'. The principle of it is illustrated in the central diagram of Text-fig. 1. The condenser having been raised above its so-called 'critical' position, the image of the pencil-point has been brought above the diatom into the front focal plane of the objective, which thus throws parallel rays from it up the tube. The eye, being focused for distant vision, receives these rays and one sees the pencil-point. The objective is now being used as an eyepiece.

Discard the pencil and put the small stop in front of the lamp. On looking through the cork you will see the stop *not filling the whole of the back lens of the objective*. We here deliberately use a stop that will not fill the back lens with light. (To prevent any possibility of misunderstanding I mention here that the iris diaphragm below the condenser must be fully open throughout the whole procedure.)

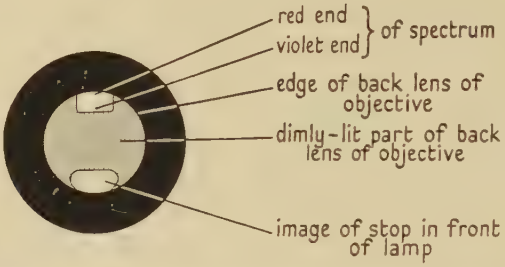
Keeping the eye at the hole in the cork, grasp the mirror in both hands and turn it in its gimbals in such a way that the circular patch of light (the visual image of the stop in front of the lamp) passes to one side of the back lens of the objective. Stop when about half of the patch of light has passed out of



TEXT-FIG. 1. Diagram showing the passage of rays of light in the microscope, with the condenser in three different positions.

view and the other half remains at the edge of the back lens. (The circle of light becomes distorted into an oval as it passes towards the edge.) Suppose you have moved the light towards the east: now bring it slowly round the edge of the back lens past the south-east towards the south. As it approaches the south, a spectrum will be seen at the edge of the back lens of the objective a little to the east of north (not to the west of north, as one might expect); this spectrum will move towards the north and will be due north when the direct light is due south. The violet end of the spectrum will be directed

towards the centre of the back lens. Now tilt the mirror to and fro to bring the light a short distance towards the middle of the back lens and back towards the edge of the back lens again, keeping it in the north–south line. Meanwhile watch the spectrum. Leave the mirror in the position that makes the spectrum as bright and complete as possible. The more of the red end of the spectrum you can bring within the back lens, the better. Text-fig. 2 shows the appearance that should be obtained. (If a glass mirror be used, there will be faint additional images of the stop, not quite coinciding with the bright image.)



TEXT-FIG. 2. Diagram showing the appearance seen on looking through the hole in the cork, when the direction and degree of obliquity of the light are correct.

Remove the cork and place the high-power eyepiece in position. The striations of the diatom will now be clearly resolved. There is no need whatever to use a colour-screen, even with a non-apochromatic objective. If perfection is desired, focus carefully with the fine adjustment, replace the eyepiece by the cork, and make very small movements of the mirror until the best possible spectrum is obtained; then replace the eyepiece.

In whatever direction the diatom happens to be lying, the same procedure should be adopted (with the obviously necessary changes).

Mr. W. E. Watson-Baker very kindly visited me in Oxford to see a demonstration of the method just described. He allows me to say that the striations of *Amphipleura pellucida* were resolved more quickly than by any other method he has ever seen, and that the images produced were superior to any obtainable by an oblique-light stop placed below the condenser. These comments are mentioned in the hope that the opinion of such an experienced microscopist will encourage others to give the method a trial.

It might be thought that the chromatic and other corrections of the objective would be upset by placing the condenser above its 'critical' position. To investigate this, I have made a careful examination of a number of species of test-diatoms, using central (not oblique) high-condenser illumination. I can find no evidence that the image is either better or worse than with 'critical' illumination. The expression 'critical' illumination is indeed a bad one, for it begs the question whether this is better than other methods. Hartridge (1919) has denied on both theoretical and practical grounds that

'critical' is necessarily preferable to other kinds of illumination: it is simply an easy and reliable way of getting good results.

If the substage iris diaphragm is somewhat closed and then kept in the same position while the two methods of illumination are tried, a larger area of the back lens of the objective will be filled with light by high-condenser than by critical illumination (provided that a sufficiently large stop is used in front of the lamp when the condenser is in the high position). When one is studying diatoms such as *Navicula lyra*, which are not well seen under full-cone illumination, this fact must be kept in mind; for otherwise there will be a danger of using too wide a cone of light when the condenser is in the high position. My attention was called to this point by Dr. O. L. Thomas, who has been kind enough to examine this new method of illumination.

One advantage of high-condenser illumination is that a perfectly structureless source of light need not be used, since the image of the light is not focused in the plane of the object; further, there cannot be any interference with the image by dust on any screen or cooling-chamber that may for any special purpose be placed between the lamp and the condenser.

The direction and degree of obliquity of the light may also be controlled by what I call 'low-condenser illumination', which is explained in the third diagram of Text-fig. 1. The condenser is here placed *below* the 'critical' position, and the source or light is thus focused below the object. An actual image of the source of light is now produced just behind the back focal plane of the objective, where it can be examined, if desired, by screwing a low-power objective into the bottom of the draw-tube. This is not nearly such an effective method for obtaining oblique illumination as that described above, but it is useful in phase-contrast microscopy. If an annular stop be put in front of the source of light, its image will be produced just behind the back focal plane of the objective, where the phase-plate may conveniently be placed.

Dr. R. Barer has kindly read this paper and given me the benefit of his criticism. He remarks that the method of illumination that I adopt is best described by saying that the aperture-stop, usually situated just below the condenser, has been removed to a considerable distance, so that the mirror lies between the aperture-stop and the condenser. I thank Mr. I. C. J. Galbraith, who gave valuable advice on an important theoretical point while I was engaged in perfecting the method of oblique illumination described in this paper.

Summary

The purpose of the method is to facilitate the resolution of extremely fine regularly-repeated markings on microscopical objects. Resolution is obtained by using the objective as an eyepiece to observe the *direction* and *degree of obliquity* of the light; these are controlled by movements of the mirror. To enable the objective to be used in this way, the condenser is raised above the

position required for so-called 'critical' illumination. The diffraction-spectrum formed by the markings on the object is clearly seen while the mirror movements are being made, and it is easy to produce a bright and complete spectrum.

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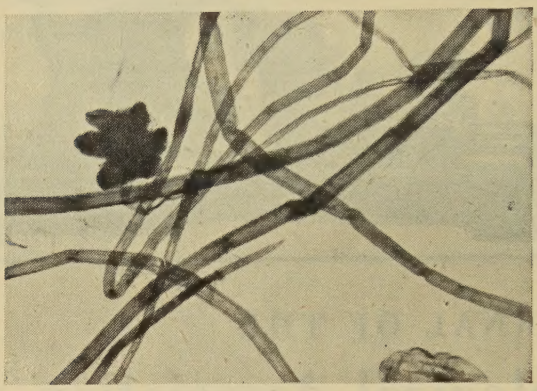

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